Anaerobic Biodegradation of Natural and Synthetic Polyesters (Anaerober Bioabbau von natürlichen und synthetischen Polyestern)

Von der Gemeinsamen Naturwissenschaftlichen
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DISSERTATION

von
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Symbols and Abbreviations

Å	Angstrom	-
A	area	[cm^2]
APS	Ammonium per-sulfate	-
AS	Anaerobic River sediment	-
Asp	Aspartate	-
ASTM	American Society for Testing Materials	-
B	1,4-Butanediol	-
BSA	Bovine serum albumin	-
BTA	Copolyester consisting of 1,4-Butanediol, terephthalic acid and adipic acid	-
BTA-S	Copolyester consisting of 1,4-Butanediol, terephthalic acid and adipic acid blended with starch	-
CEN	Comité Européenne de Normalisation	-
Ø	Diameter	[cm]
Br	Brewer´s anaerobic medium	-
cAMP	Cyclic-adenosyl-3´,5´-monophosphate	-
CAP	Catabolite activator protein	-
CCR	Carbon catabolite repression	-
CRE	catabolite-responsive element	-
d	Day	-
Da	Dalton	-
DIN	Deutsches Institut für Normung	-
DNA	Desoxyribonucleic acid	-
rDNA	Ribosomal desoxyribonucleic acid	-
DSMZ	German Culture Collection	-
DTT	Dithiothreitol	-
g	Gram	[g]
FF	Fast Flow	-
FPLC	Fast Protein Liquid Chromatography	-
GBF	National Research Center of Biotechnology (Gesellschaft für Biotechnologische Forschung, mbH)	-
GC	Gas chromatography	-
G+C	Guanidine + Cytosine	-

Gly	Glycine	-
GMB	Glucose-vitamin-mineral-medium	-
GPC	Gel permeation chromatography	-
GV	DSM mediun number 500	-
h	Hour	-
HIC	Hydrophobic Interaction Chromatography	-
His	Histidine	-
HPr	heat-stable phosphocarrier protein of the phosphotransferase system	-
l	Liter	-
ICI	Imperical Chemical industry	-
ISO	International Standardization Organization	-
lac	Lactose	-
LS	Laboratory Sludge	-
LSS	Laboratory Sludge Supernatant	-
Δm	Change in weight	[g]
M_0	Initial weight of polymer film	[g]
mbar	Millibar pressure	-
min	Minute	-
Mol	Mol	-
M_n	Weight average molecular number	[$g.mol^{-1}$]
M_w	Weight average molecular weight	[$g.mol^{-1}$]
MSV	Mineral Salt Vitamin medium	-
MSV-LSS	Mineral Salt Vitamin medium supplemented with Laboratory sludge supernatant	-
n	Number	-
nd	Not detected	-
PAGE	Poly-acrylamide Gel Electrophoresis	-
PCL	Poly(ε-caprolactone)	-
PCL-S	Poly(ε-caprolactone)–starch blend	-
PEP-PTS	Phosphoenolpyruvate-dependent sugar transporting phosphotransferase system dependent sugar transporting phosphotransferase system	-
PHA	Polyhydroxyalkanoates	-
PHB	Poly(β-hydroxybutyrate)	-

PHBV	Poly(β-hydroxybutyrate-co-β-hydroxyvalerate)	-
PVC	Poly(vinylchloride)	-
PY	Peptone - yeast extract medium	-
PYG	Peptone-yeast extract-glucose medium	-
RAAM	Revised Anaerobic Mineral Medium	-
RNA	Ribonucleic acid	-
rpm	Round per minute	-
SDS	Sodium-dodecyl sulfate	-
SEM	Scanning Electron Microscopy	-
Ser	Serine	-
SP	Saturated polyester	-
t	Time	[min]
T	Terephthalic acid	-
T_m	Melting temperature	[°C]
ThBiogas	Theoretical biogas	-
TBW	Thermophilic Biowaste	-
TEMED	N,N,N´N´-Tetramethylethylendiamin	-
TG	Thioglycolate medium	-
TVLS	Anaerobic TVLS-medium	-
UV	Ultra violet	-
V	Volume	[l]
WWS	Waste Water Sludge	-
X	Variable for any given amino-acid	-

1. Introduction

Synthetic polymers – designated as plastics - have become technologically significant since the 1940s and since then they have come to replace glass, wood, masonry and other constructional materials, and even metals in many industrial, domestic, commercial and environmental applications (CAIN, 1992). These widespread applications are not only due to their favorable mechanical and thermal properties but mainly due to stability and durability of plastics. On the other hand, plastics also play an important role for many “short live” applications such as packaging and commodity as well as hygienic products which represent the major part of plastic waste (WITT ET AL., 1997). Because of their persistence in the environment, the increased costs of solid waste disposal (owing to the reductions in available landfill space), as well as the potential hazards from waste incineration (such as dioxin emission from PVC incineration), plastics became more and more a waste deposit problem.

Consequently, the past two decades have witnessed a growing public and scientific concern regarding the use of biodegradable materials as an ecologically probably useful alternative to conventional plastics offering a solution for the existing grave problem of plastic waste (BICHLER ET AL., 1993). Biodegradable plastics do neither contribute to plastic litter nor - if made of renewable resources - lead to the depletion of finite resources. Current research interest in biodegradable plastics is connected with well defined areas of use. A number of biodegradable plastics – mostly biodegradable polyesters - have indeed been successfully developed over the last few years to meet the specific demands in various fields and industries (SASIKALA AND RAMANA, 1996; AMASS ET AL., 1998). These materials may offer one solution to managing packaging waste. But there are also agricultural uses, such as the controlled release of fertilizers and pesticides, applications in the automotive industry and as surfactants.

Biodegradable plastics opened the way for new considerations of waste management strategies since these materials are designed to degrade under environmental conditions or in municipal and industrial biological waste treatment facilities. Aerobic composting as well as anaerobic biogasification of waste are currently in use, especially in European countries, and the latter process is becoming more and more established because of the added benefit of energy conservation due to biogas recovery. Most of the plastics on the market, claimed to be biodegradable, are based on synthetic and microbial polyesters (Augusta ET AL., 1992; WITT ET AL., 1997). Polyesters are potentially biodegradable due to the hydrolysable ester

bonds. In addition, they combine several properties that make them attractive candidates for various industrial applications. However, the variation in polyester properties of microbial sources such as poly(hydroxybutyrate) (PHB) is limited. This situation necessitates the use of synthetic biodegradable polyesters with a great spectrum of mechanical and thermal properties ranging from poly(ε-caprolactone) with good biodegradability but restricted applicability due to its low melting point to special aliphatic-aromatic copolyesters (e.g. Ecoflex®) with good material properties and biodegradability (WARZELHAN ET AL., 1996; WITT, 1996; WITT et al., 1997).

The best understood and most extensively studied biodegradable plastics with regard to biodegradation are aliphatic polyesters, especially the bacterially produced poly(hydroxyalkanoates) (PHAs) (ANDERSON AND DAWES, 1990; DOI ET AL., 1990; AUGUSTA ET AL, 1993; MARCHESSAULT ET AL., 1994; JENDROSSEK ET AL., 1996; MERGAERT ET AL., 1996A). Research on polyester degradation has more or less exclusively concentrated on aerobic systems (MERGAERT AND SWINGS, 1996; SUYAMA ET AL., 1998) and various extracellular depolymerases from aerobic bacteria responsible for primary polyester depolymerization have been isolated and investigated. The work on such extracellular depolymerases is reviewed by BRANDL ET AL. (1995) and JENDROSSEK (1998).

The evaluation of the anaerobic breakdown of plastics, however, is still in a developing stage and only few reliable investigations are available. Yet, the anaerobic decomposition is of particular importance, e.g. with regard to the biodegradation in landfills (where anoxic conditions exist) as well as anaerobic waste treatment processes.

The complete mineralization to methane and CO_2 of the polymer under anaerobic conditions involves successions of syntrophic associations (GOTTSCHALK AND PEINEMANN, 1992) and consequently most studies published focus on mixed and unspecified populations such as diverse anaerobic sludges and/or sediments (BUDWILL ET AL., 1992; PÜCHNER, 1995; URMENETA ET AL.; 1995, REISCHWITZ ET AL., 1998). Investigations using individual cultures were restricted to the degradation of poly(ß-hydroxybutyrate) (PHB) by a Gram negative obligate anaerobic bacterium *Ilyobacter delafieldii* (JANSSEN AND HARTFOOT, 1990; JANSSEN AND SCHINK, 1993). (Contrarily, a total of 695 strains of aerobic PHB-degrading microorganisms are currently identified (MERGAERT AND SWINGS, 1996)). Anaerobic investigations with defined cultures as well as anaerobic biodegradability tests for other plastics are still missing.

2. Aim of work

The traceable proof of the complete biodegradability even under anaerobic conditions is a prerequisite for the environmentally safe application of biodegradable polyesters. Thus, the aim of the present work was to partly overcome the existing gap in knowledge of anaerobic biodegradation of plastic materials. The main question to be answered was whether or not synthetic polyesters -beside the natural ones- are biodegradable under anaerobic conditions. No studies on the anaerobic degradability of the aromatic-aliphatic copolyester BTA 40:60 (or its aliphatic counterpart SP 4/6) have been reported. However, clarification of its biodegradability under anaerobic conditions is needed because the production of this polymer has already been started on an industrial scale.

In order to gain more insight in the anaerobic polyester biodegradation processes, the present investigation focused first on the evaluation of the biodegradability (fig. 2.1) of technically relevant natural and synthetic aliphatic polyesters as well as a synthetic statistical aromatic-aliphatic copolyester using mixed undefined anaerobic bacterial populations. Using a broad and versatile spectrum of anaerobic organisms, it had to be clarified to what extent anaerobic biodegradability can be expected in different anaerobic habitats such as technical and managed waste disposal systems or a natural environment.

Although degradation tests with mixed cultures are practice oriented and realize naturally occurring conditions, they are usually limited by reproducibility and are not suitable to elucidate the biochemical mechanism. Consequently, in a second stage, it had to be clarified, if all the polyesters under test are depolymerizable by anaerobic single strain cultures (in addition to sludges and sediments). Consequently, individual anaerobic monocultures capable of depolymerizing these polyesters had to be screened and eventually isolated.

Beside gaining information about the occurrence and versatility of polyester depolymerizing anaerobes, selected strains can be characterized and used in defined degradation tests. The availability of pure isolates opens opportunities for more detailed investigations through reproducible and systematic test procedures on the anaerobic degradation process. In fact, biological degradation of polymers is generally influenced by a number of factors. Beside the nature of the polymeric substance, the kind of organism involved in biodegradation and the environmental conditions (e.g. nutrient supply, temperature, pH, etc.) are known to drastically influence the degradation rate. It was, therefore, aimed to identify the factors

influencing the anaerobic biodegradability of the polyesters under test with the anaerobic isolates.

Furthermore, the development of an appropriate anaerobic test method to determine the biodegradation of a polyester under defined conditions and within a reasonable time period using a characterized single strain culture was envisaged. Trying to understand the mechanisms by which polymers are degraded, finally the microbial anaerobic depolymerizing enzyme should be isolated and characterized.

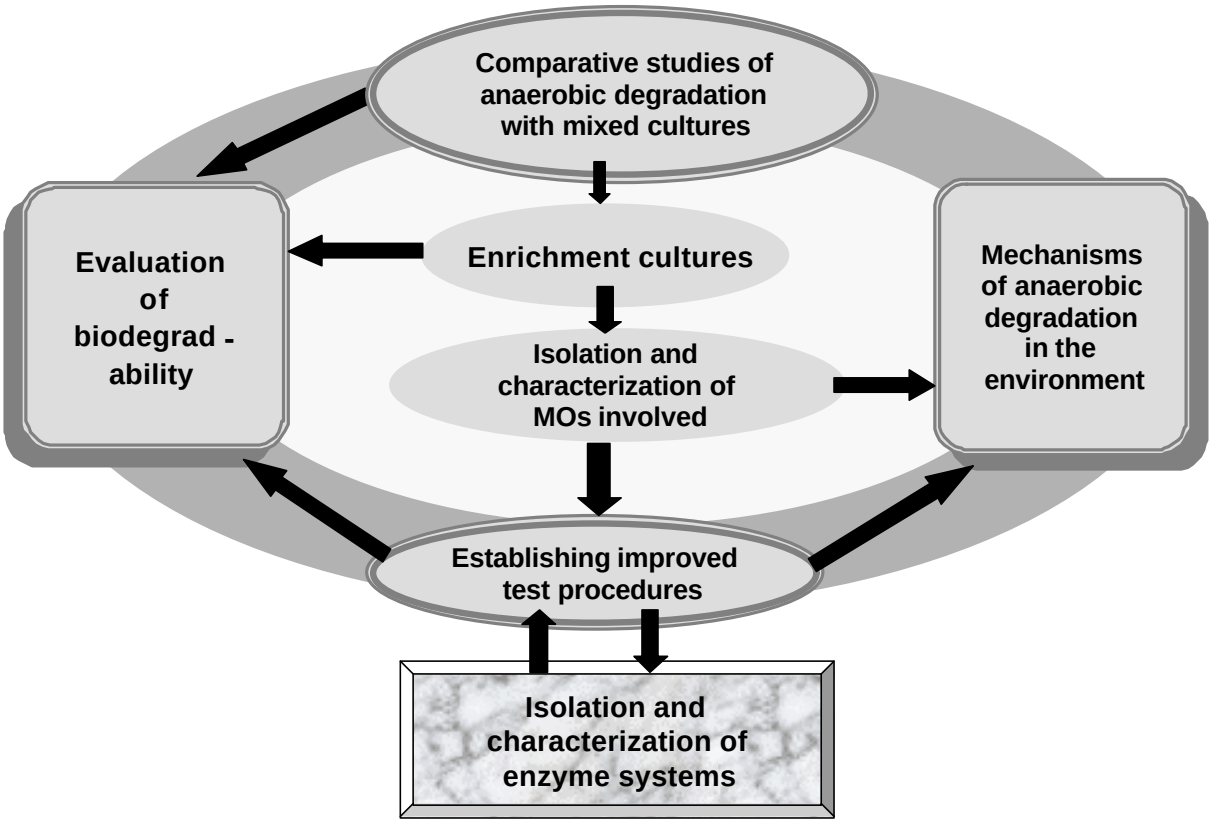


Fig. 2.1. Schematic representation of the main goals of the present work.

3. Theoretical Background and Literature Review

3.1. Biodegradable polymers

The current worldwide demand for plastics is in excess of 100 million tones per year (RAPRA TECHNOLOGY LIMITED, 1992). The rapid growth in consumption of plastics in recent years has lead to concerns from consumers, environmentalists and indeed the plastic industry, regarding the effective management of post consumer waste and greater use of, and dependence on, fossil fuels. The emphasis now is on minimizing the unnecessary use of plastics and on developing methods of recovery and recycling. Alongside and compatible with these, much work is being carried out into different ways to reduce the environmental impact of plastics. One way of doing this could be the use of biodegradable plastics.

Generally, conventional technical and synthetic polymers such as polyethylene and polystyrol are not biodegradable (AMINABHAVI ET AL., 1990). On the other hand, biodegradable polymers are expected to resist microbial attack for the period of use and they should decompose once the material is no longer needed. Natural biodegradable polymers like protein, cellulose, starch, lignin and even the natural polyester PHB, have a backbone of carbon atoms interrupted by hetero-atoms such as nitrogen and oxygen in the main polymer chain. These heteroatoms represent points of attack for enzymatic hydrolytic and oxidative cleavage (TIMMINS AND LENZ, 1994). Generally, polymers which contain double bonds or ether, ester and peptide bonds in the backbone such as natural rubber, polyethers, polyesters and polyamides, respectively, are more or less biodegradable. However, one exception of a biodegradable polymer with pure C-C bonds is poly(vinylacohol) (PVOH). In this case, degradation proceeds via primary oxidation of the hydroxyl groups, followed by polymer chain cleavage similar to fatty acid degradation (SAKAI ET AL., 1986).

In the 1970s, work was started in US and elsewhere to produce photo-degradable and biodegradable plastics for the packaging industry. The requirements for such polymers were:

(1) Non toxic materials with non-toxic degradation products that would not affect the drainage water from landfills;
(2) Polymers with suitable mechanical properties for specific uses;
(3) Economic viability;
(4) Degradation control of the plastics via polymer modification; and
(5) Processability

A vast range of biodegradable polymers are at present available and summarized in Table 3.1.

Table 3.1. Biodegradable polymers (AMASS ET AL., 1998).

Type	Comments	Examples
Polyesters	Formed by condensation, ring-opening polymerization or bacterial synthesis	Poly(α-ester)s, polylactones and poly(β/γ-ester)s.
Polyamides	Only structurally modified synthetic polyamides are biodegradable	Hydroxylated nylon
Polyurethanes	Only structurally modified synthetic polyurethanes are biodegradable	Hydrophilic ether urethanes
Polyureas	Virtually “non”-degradable	Urea formaldehyde
Polyethers	Dissolve if carbon chain is short; but also found to degrade	General formula $-(-O-(CH_2)x-)_n$ Poly(ethylene oxide) (PEO), $x = 2$.
Polyanhydrides	Degradation thought to be mainly by surface erosion	Poly(bis(p-carboxyphenoxy)) alkane anhydride or PCPX.
Poly-(orthoester)s	Degradation thought to be mainly by surface erosion	Poly(3,9-bis(ethylidene-2,4,8,10-tetraoxaspiro[5,5])undecane-co-hexane diol) or DETOSHU-HD
Polypeptides or proteins	Naturally occurring polyamides containing amino acid units	$-(-C(R)-OH-CO-NH-)_n$ with different R groups and chain lengths, e.g. natural proteins collagen, gelatin
Polysaccharides	Basic sugar units joined by glycoside linkages; hydrolyzed abiotically and by enzymes	Naturally occurring starches and different forms of celluloses.

The source of these materials is mainly of two types: natural materials known to biodegrade and synthetic biodegradable polymers. The so called biodegradable polymers can, therefore, be classified into four main groups after WITT ET AL. (1997) as follows:

1) natural polymers
2) chemically modified natural polymers
3) synthetic polymers composed from natural building blocks
4) synthetic polymers from petrochemical building blocks.

Table 3.2 lists the advantages and disadvantages of each group giving examples for each. Synthetic and microbial polyesters form the greatest number of reported types of biodegradable polymers and are the main focus of the present study.

Table 3.2. Classification of the biologically degradable polymers based on the polymer source after WITT ET AL. (1997).

Source of polymer	Advantages	Disadvantages	Examples
Natural polymers	Renewable resources, mostly low-priced	Low reproducibility and variability of material properties	Starch, cellulose, PHB
Modified natural polymers	Partially from renewable resources, variability of material properties	Expensive, structure control is difficult	Cellulose acetate, starch acetate
Fermentatively produced monomers	Renewable resources, good reproducibility of material properties and structure control	Expensive; (exception: PLA; price = 3 DM/kg)	PLA
Petrochemically produced monomers	Good material properties, good reproducibility of material properties, inexpensive, fast synthesis	No renewable resources	Polyester amides, polyester urethanes, aliphatic homopolyesters, aliphatic-aromatic copolyesters

Since only the polymer structure and not the source of the monomeric building blocks is responsible for biodegradability (WITT ET AL., 1999), precise demands on polymer structure

of biodegradable polyesters are considered nowadays, as summarized by CHANDRA AND RUSTIGO (1998)

- Polymer structure:
  -The presence of hydrolytic linkages along the polymer chain, representing enzymatically cleavable bonds within the polymer chain
- Interaction of the active site of the enzyme with the polymer must be possible through:
  -The stereochemistry of the polymer;
  -The hydrophilic-hydrophobic character of polymers;
  -The flexibility of polymer chains;
- Polymer morphology:
  -The length of the repeating units which in turn affects the degree of cristallinity;
  -The size, shape and number of crystallites in semicrystalline polymers;
  -Amorphous regions are more rapidly degraded that crystalline ones.
- Molecular weight
  - Biodegradation is favored by low molecular weights

Current research interest in biodegradable polymers is connected with well-defined areas of use. Biodegradable plastics offer one solution to managing packaging waste. However, biomedical applications of biodegradable and biocompatible polymers generate an enormous amount of research interest. Uses in this field range from medical to industrial applications (Table 3.3).

Table 3.3. List of practical applications of PHA after SASIKALA AND RAMANA (1996).

Medical applications
1) Surgical pins, sutures, staples, swabs, and wound dressing
2) Blood vessel replacement
3) Bone (orthopedic) replacements and plates
4) Stimulation of bone growth by piezoelectric properties
5) Biodegradable carrier for long-term dosage of drug and medicines
Industrial applications
1) Biodegradable carrier for long-term dosage of herbicides, fungicides, insecticides, or fertilizers
2) Packing containers, bottles, wrappings, bags and films, fiber-reinforced biodegradable bicycle helmet, autoseparative filter
3) Disposable items such as diapers or feminine hygiene products

More detailed information are given by market studies of WESTERHAUSEN ET AL. (1990), FRITZ ET AL. (1994) as well as WITT ET AL. (1997).

3.2. The biological degradation

Biodegradation as opposed to chemical or physical waste treatment has the advantage of being an autoregulatory degradation process and does not need to be controlled in any way. The compost heap is an example of such a microbial waste degradation facility that is easy to maintain and produces a useful raw material from wastes through an enormous variety of degradative activities. In contrast to the compost heap, landfills are nearly entirely anoxic habitats containing a complex microbial community, including fermentative, methanogenic, and sulfate-reducing bacteria (BARLAZ ET AL., 1989; SMITH ET AL., 1990). These microbial consortia degrade the various substrates mainly by fermentation, usually coupled to methane formation as the terminal process. The limits and principles of anaerobic degradation activities have been summarized by ZEHNDER AND STUMM (1988) as well as SCHINK (1988).

3.2.1. Defining biodegradability

Unfortunately, the term biodegradation has not been applied consistently, resulting in confusion. Deterioration or a loss in physical integrity of a material is often mistaken for biodegradation. Biodegradation, however, is a natural and complex process of decomposition facilitated by biochemical mechanisms.

There are two definitions describing biodegradability according to the fate of the polymer (BUCHANAN ET AL., 1993; BATTERSBY ET AL., 1994):

Primary biodegradability: (or partial biodegradability) is the alteration in the chemical structure resulting in a loss of specific polymer properties.

Ultimate biodegradability: (or total biodegradability) deals with total mineralization and assimilation. The material is totally degraded by microorganisms with production of carbon dioxide (under aerobic conditions) or methane (under anaerobic conditions), water, mineral salts and biomass (AUGUSTA ET AL., 1992; PALMISANO AND PETTIGREW, 1992).

However, two other definitions are important for the biodegradation of organic compounds (SEAL, 1991).

Ready biodegradable: is assessed in stringent tests which provide limited opportunity for biodegradation and acclimatization to occur.

Inherent biodegradable: is assessed in tests based on a prolonged exposure of the test compound or other conditions favoring biodegradation. The degradation occurring under optimized conditions must not necessarily occur under normal test conditions.

In the present work the definitions of *biodegradation* as well as *biodegradability* for plastics according to standardized test methods (MÜLLER, 1994) and DIN 54900-2 (1998) are used, since they are the most stringent ones among the definitions laid down by ASTM, CEN, and ISO (Table 3.4):

Biodegradation: A process induced by biological activity which results through the change of the chemical structure of the material in naturally occurring metabolic products.

Biodegradability: A plastic material is biodegradable if all of its organic constituents are subject to complete biological degradation. The environmental conditions as well as the degradation rates are determined by standardized methods.

Table 3.4. General definitions of a biodegradable polymer (or plastic) proposed by Standard Authorities and summarized by CALMON-DECRIAUD ET AL. (1998).

Standard Authorities	Biodegradable plastics
ISO 472-1988	A plastic designed to undergo a significant change in its chemical structure under specific environmental conditions resulting in a loss of some properties that may vary as measured by standard test methods appropriate to the plastic and the application in a period of time that determines its classification. The change in the chemical structure results from the action of naturally occurring microorganisms.

Standard Authorities	Biodegradable plastics
ASTM sub-committee D20-96	A degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria and fungi.
DIN 103.2-1993 German working group	A plastic material is called biodegradable if all its organic compounds undergo a complete biodegradation process. Environmental conditions and the rates of biodegradation are to be determined by standardized methods.
CEN (May 1993)	A degradable material in which degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide and/or methane and new cell biomass.
Japanese Biodegradable Plastic Society (1994)	Polymeric materials which are changed into lower weight compounds where at least one step in the degradation process is through metabolism in the presence of naturally occurring organisms.

In addition PAGGA (1994) and WITT ET AL. (1997) survey important standardized test methods.

3.3. Microbial degradation under anaerobic conditions

Obligate anaerobic bacteria are the simplest in structure and biochemistry and are the most closely related to the earliest forms of life (LOWE ET AL., 1993). The anaerobic breakdown of organic matter is recognized as a complex process involving the coordinate activity of a number of different bacterial trophic groups (MCINERNY ET AL., 1980; GUJER AND ZEHNDER, 1983; ZINDER, 1984). Many of the species involved exhibit slow growth rates and require strict anaerobic conditions for cultivation or, as in the case of syntrophic bacteria, cannot be cultivated in monoculture.

The possible scheme for anaerobic food CHAINS (MCINERNY AND BRYANT, 1981; VOGELS ET AL., 1988), as they occur in nature in the absence of nitrate, nitrite, mangan, iron and sulfate as electron acceptors are presented in Fig 3.1.

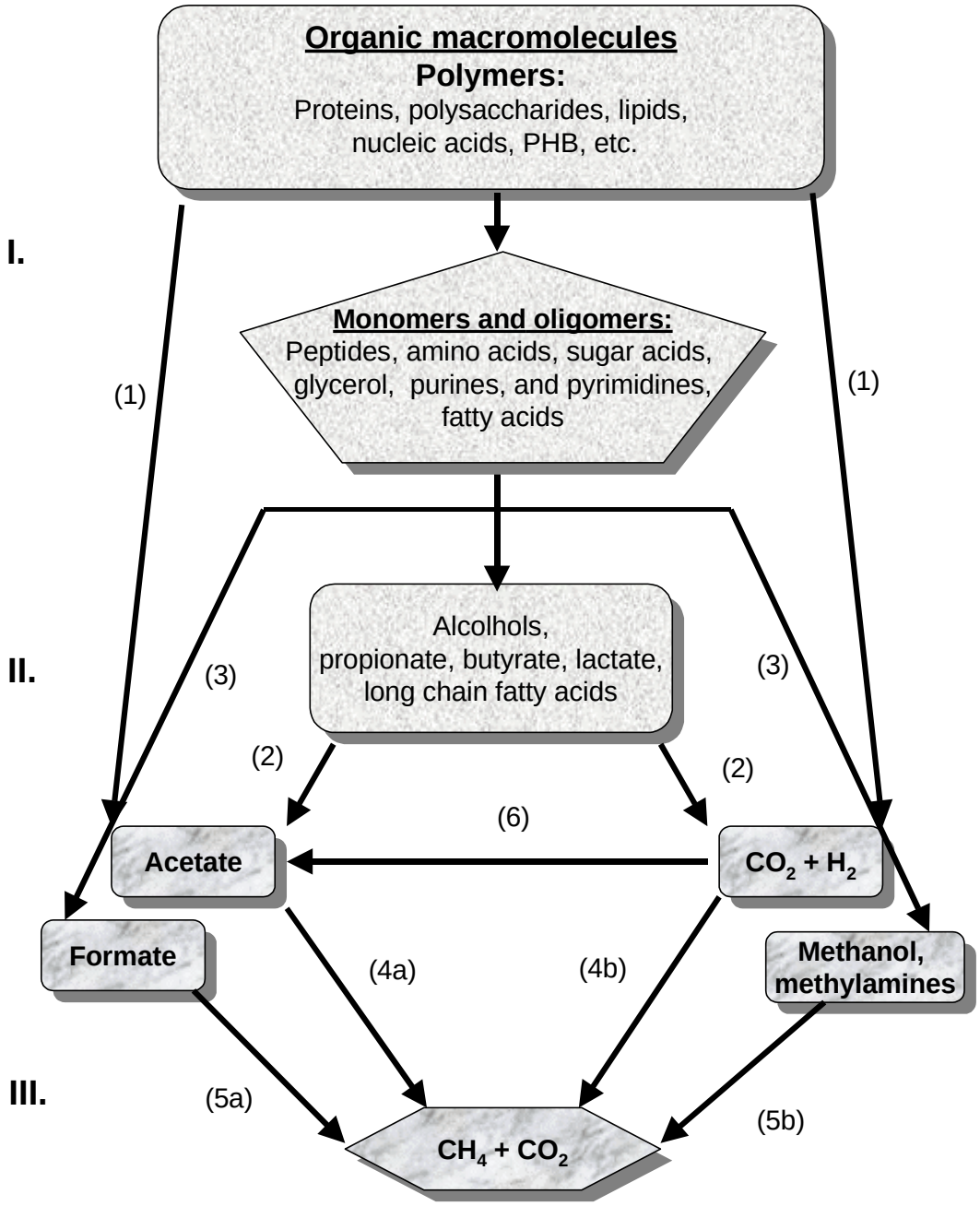


Fig. 3.1. Three-stage scheme for complete anaerobic degradation of organic matter showing sequence and major metabolic groups of bacteria: (1) hydrolysis and fermentation of polymers by fermentative bacteria; (2) acetogenic dehydrogenation by obligate H_2-producing (proton reducing) acetogenic bacteria; (3) acetate decarboxylation by obligate H_2- producing (proton reducing) acetogenic bacteria: (4) acetate decarboxylation (4a) and reductive CH_4 formation (4b) by H_2-oxidizing methanogenic bacteria; (5) methanol and methylamines also serve as methanogenic substrates; and (6) acetogenic hydrogenation by acetogenic bacteria. (MACKIE ET AL., 1991; modified).

In the first stage, fermentative bacteria hydrolyze (with the aid of extracellular hydrolases) and ferment carbohydrates, proteins, and lipids as well as other macromolecules with the

production of propionate, alcohols and long chain fatty acids, but also acetate, H_2 and CO_2 (GOTTSCHALK, 1979). These compounds (with the exception of acetate, H_2 and CO_2) are subsequently degraded by a second group of bacteria called the obligate H_2-producing (proton-reducing) acetogenic bacteria. Finally, methanogens reduce CO_2 to CH_4 using H_2 produced by other bacteria, and they also cleave acetate to CH_4 and CO_2. The relative importance of H_2 and formate as methanogenic substrates involved in interspecies hydrogen transfer has not been clearly resolved (BOONE ET AL., 1989). A fourth group of bacteria is able to carry out acetogenic hydrogenation producing acetate from H_2 plus CO_2. Hereby, bacteria of different groups are involved in a close symbiosis (SAHM, 1981). The methanogenic bacteria need the hydrogen produced by acetogenic and fermentative bacteria and prevent their being inhibited by high hydrogen concentration.

The rate limiting step in the anaerobic degradation pathways is generally considered to be acetogenesis (MUDRACK AND KUNST, 1991). However, for polymeric molecules, which include biodegradable materials (and plastics), hydrolytic breakdown of polymers by fermentative bacteria appears to be the limiting factor (ZACHÄUS, 1995; GARTISIER ET AL., 1998).

3.4. The anaerobic degradation of a polymer

The biological degradation of water insoluble polymeric substances is a complex process involving several subsequent steps induced by the action of enzymes. The most important type of enzymatic polymer cleavage reaction is hydrolysis (SCHINK ET AL, 1992). Especially glycosidic bonds, but ester and peptide linkages as well, are subject to hydrolysis through nucleophilic attack on the carbonyl carbon atom. Polysaccharides, fats, PHB, gelatin, keratin etc., but also the synthetic polymers polylactate or polymalate (HUTCHINSON AND FURR, 1987) are all degraded through such reactions. Generally, the biological degradation of polymeric substances is influenced by the:

- Presence of enzymes and microorganisms:
  -Induction of the synthesis of the degradative enzyme by the polymer
  -Optionally, a constitutively secreted enzyme
- Biotic availability of the polymeric structure:
  -Cristallinity of the polymer
  -Accessibility of the cleavable bond to the enzyme
  -Formation of microbially metabolizable products due to the enzymatic cleavage. (The

products of enzymatic cleavage should be similar and not necessarily identical to chemicals found in nature.)

- Abiotic factors:
  -Appropriate environment: such as the presence or absence of oxygen, the availability of nutrients, adequate pH values, temperature, etc.

The high molecular weight and generally non soluble polymers cannot be taken up into the microbial cell. Therefore, polymer degrading hydrolases are excreted by the producing microbial cell into the surrounding milieu to allow the direct contact with the polymeric substrate. The polymer chains are then cleaved until short chained water soluble products are produced which can be transported through the cell membrane. Inside the cell, these degradation products are intracellularly metabolized into water, CO_2 and biomass and others (Fig. 3.2).

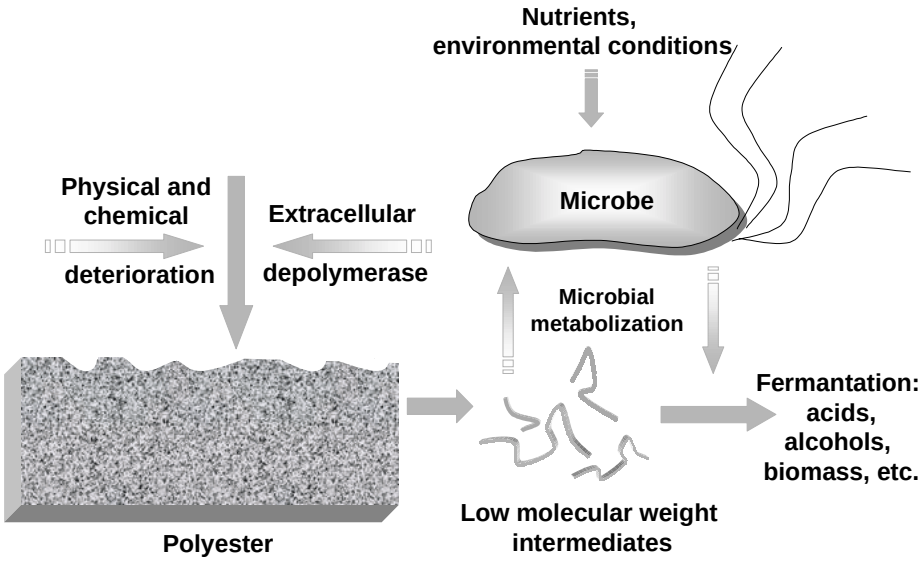


Fig. 3.2. Schematic representation of the microbial degradation of polyesters under anaerobic conditions.

For self-regulation of a biodegradation process, it is important that the microorganisms catalyzing it have some direct or indirect advantage from it. There will be no positive increase of activity with increasing polymer availability if the polymer-degrading (e.g. hydrolase-producing) bacteria cannot make any use of the monomers produced. However, sometimes the hydrolase producing organism may not directly benefit from the primary degradation process. In this case other organisms use these degradation products for cell metabolism and may in one way or the other symbiotically interact with the polymer hydrolyzing organism.

3.5. Isolation and selection of usable anaerobic bacterial strains for polyester biodegradation investigations

The principal route for screening a polyester hydrolyzing microorganism is shown in fig. 3.3. The potential biological system can be analyzed in different steps of the screening program resulting in the selection of a mono culture via mixed cultures and finally pure stable and active strains. Eventually, a useful biocatalytic system for further mechanistic investigations is obtained.

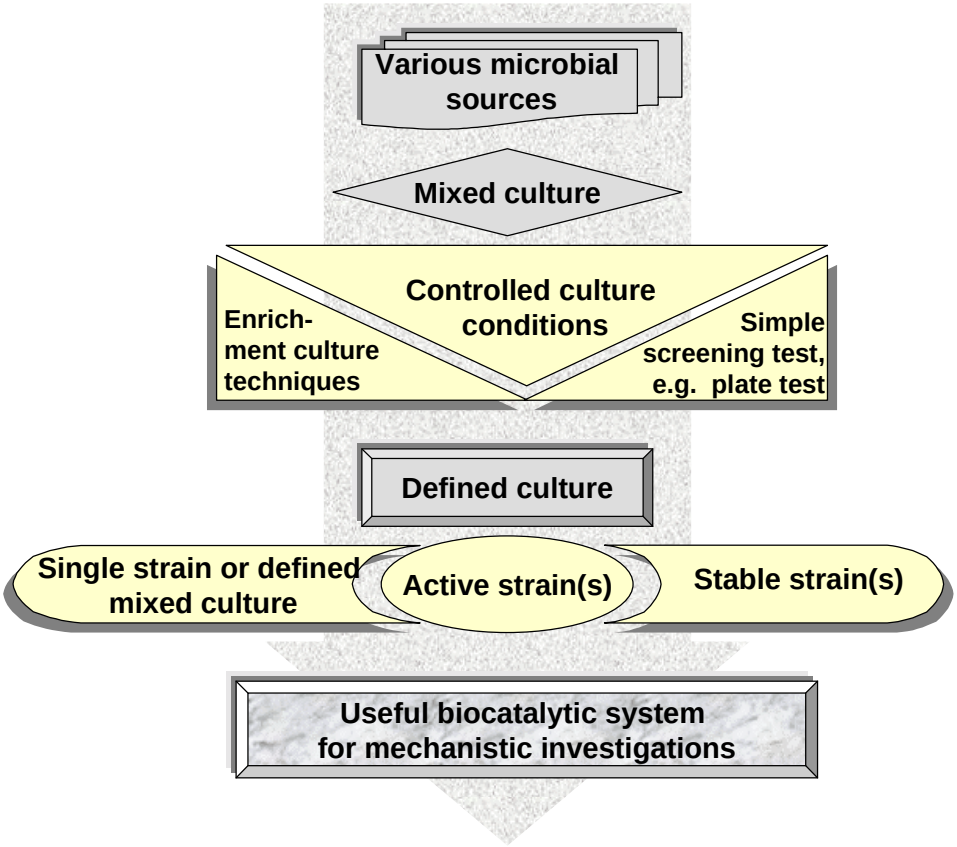


Fig. 3.3. The principal route for screening a polyester hydrolyzing organism.

The principle of enriching the desirable target organisms from the various kinds of organisms that coexist in a habitat was first developed by Winogradsky and Beijerinck (SCHLEGEL, 1992; BROCK AND MADIGAN, 1991). For successful isolation of a given organism into pure culture, the organism generally must comprise a sufficiently high proportion of the mixed population. Enrichment methods are designed to achieve an increase in the relative numbers of a particular organism by favoring growth, survival (i.e. physiological competition), or its spatial separation from other members of the population.

This method was previously successfully applied for the isolation of aerobic organisms capable of depolymerizing different polyesters such as poly(ε-caprolactone), poly(ß-hydroxybutyrate), Poly(ethylenesuccinate) and poly(carbonate) and the synthetic aliphatic-aromatic copolyester BTA 40:60 (BENEDICT ET AL., 1983; JENDROSSEK, ET AL., 1993B; TANSENGCO AND TOKIWA, 1998; SUYAMA ET AL., 1998; PRANAMUDA ET AL., 1999; KLEEBERG ET AL., 1999).

The selection criterion for polyester degradation is usually clear zone formation on agar plates containing the polyester of interest. The presence of clear zones proves the secretion of extracellular polyester depolymerizing enzymes by the selected target organism. The complete metabolization of the depolymerization products must be tested separately.

3.6. Polyester cleaving enzymes

Of special interests for the present work are specific hydrolases, namely the serine hydrolases (according to the classification of LEHNINGER, 1987), which include lipases, esterases, PHB-depolymerases and serine endopeptidases (WEBB, 1992). A common feature for the serine hydrolases is the presence of a specific sequence Gly-X-Ser-X-Gly (ANTONIAN, 1988; BRADY ET AL., 1990; JENDROSSEK ET AL., 1995; SCHIRMER ET AL., 1995; ARPIGNY ET AL., 1998). The catalytic mechanism of these enzymes is very similar and the catalytic center consists of a triade of serine, histidine, and aspartate residues and other oxy-anion stabilizing rest groups (KAZLAUSKAS AND BORNSCHEUER, 1998). SCHIRMER AND COWORKERS (1995) demonstrated that serine from this sequence Gly-X-Ser-X-Gly- in the active center attacks the ester bond nucleophilically.

The enzymatic degradation of a polymer by hydrolysis is a two-step process, in which the enzyme first binds to the polymer substrate and then catalyzes a hydrolytic cleavage. This initial attack on the polymer can occur by one of two modes of attack, known as *exo*- and *endo*-attack, which are distinguished by both the locus on the polymer at which a bond is cleaved and the by-products that result.

The *exo*-attack occurs strictly at the polymer chain terminus, often with a preference for one chain end moiety over the other (e.g. the hydroxyl end of a hydroxy-acid polyester rather than the carboxylic acid end), with exclusively small oligomers or monomers as the products.

On the contrary, the *endo*-attack can, in principle, occur at any location along the length of the polymer chain, and in that case a mixture of low molecular weight products results. Repeated *endo* cleavage, therefore, reduces the molecular weight of the polymer more quickly, whereas repeated *exo* cleavage results in a rapid generation of small metabolizable monomers, dimers, etc. (HUANG, 1989). In some systems, such as in the degradation of cellulose, both modes operate in synergy (TIMMINS AND LENZ, 1994).

3.6.1. PHB depolymerases

The properties of aerobic extracellular polyhydroxybutyrate (PHB) depolymerases have been extensively studied. Analysis of their genes revealed that the enzymes have a bifunctional organization composed of two domains and a linker region (MUKAI ET AL, 1993B; DOI ET AL, 1994; JENDROSSEK ET AL., 1996; KASUYA ET AL., 1999). One of the domains plays the role in binding to the solid PHB and is called the substrate binding domain. The other domain is a catalytic domain and contains the catalytic machinery composed of a catalytic triad (Ser-His-Asp). The serine is a part of the lipase box pentapeptide (Gly-X-Ser-X-Gly), which has been found to in all known serine hydrolases, such as lipases, esterases, and serine proteases (JAEGER ET AL., 1995; JENDROSSEK ET AL., 1995). The oxygen atom of serine side chain is the nucleophil that attacks the ester bond and is supported by the imidazol ring of the histidine. The positive charge of the latter is stabilized by the carboxylate group of the aspartate. In addition, the two domains are connected by fibrinectin type III or threonine-rich linker (JENDROSSEK ET AL., 1995). It has been found, that the adsorption of PHB depolymerase to the surface of PHB granules acts independently of the catalytic domain. Hence, the binding of the surface binding domain to a substrate is necessary but not a sufficient condition for the degradation. It was also suggested that the binding specificity of the substrate binding domain is broad compared with the substrate specificity of the catalytic domain (KASUYA ET AL., 1999).

3.6.1.1. Biochemical properties of PHA depolymerases

As far as has been tested, PHA depolymerases share several biochemical properties as reviewed by JENDROSSEK (1998): (1) the M_r is relatively small (below 100 kDa; for many PHA depolymerases between 40 and 50 kDa); (2) PHA depolymerases do not bind to anion exchangers but have a pronounced affinity to hydrophobic materials; (3) the pH optimum is in the alkaline range (7.5 – 9.8). Only the depolymerases of *Pseudomonas picketti* and *Penicillium funiculosum* have pH optima at 5.5 and 6.0, respectively; (4) most PHA depolymerases are inhibited by serine esterase inhibitors such as diidopropyl-fluoryl-phosphate or acylsulfonyl compounds, which have been shown to bind covalently to the

active site serine of serine hydrolases; (5) while many PHA-degrading bacteria apparently contain only one depolymerase, *Pseudomonas lemoignei* has six depolymerases, which differ slightly in their biochemical properties. It is therefore likely, that other bacteria also have more than one depolymerase. In contrast to most other bacterial depolymerases the depolymerases of *P. lemoignei* are glycosylated and contain N-acetylglucosamine and glucose. Glycosylation is not essential for activity but may improve the resistance of the extracellular enzyme to elevated temperature and/or hydrolytic cleavage by proteases of competing microorganisms, indicating an exo-type mechanism.

Depending on the depolymerases the hydrolysis products are monomers (*Comamonas* sp.) or oligomers (mono- to trimers), as in the case of other depolymerases (*Pseudomonas* sp.). In a second step the oligomers are hydrolyzed to monomers by extracellular or intracellular oligomer hydrolases.

Evidence exists that synthesis and secretion of PHB depolymerase are subject to a double regulatory control (see 3.6.3.): by derepressing elicited in the absence of a utilizable substrate and by catabolite repression in the presence of a more readily utilizable substrate (JENDROSSEK ET AL., 1993B).

3.6.2. Differences between lipases and depolymerases

It was found that the x1 residue was leucine in PHA depolymerases instead of a histidine in bacterial lipases. However, none of the PHA depolymerases shows significant lipase activity. The depolymerases are unable to (1) bind a long-chain triacylglycerol or (2) hydrolyze the lipase substrate (JAEGER ET AL., 1995). On the other hand, several lipases hydrolyze polyesters of ω-hydroxyalkanoic acids such as PCL and BIONOLLE. The presence of alkyl side chains in a polyester inhibits or at least drastically reduces its suitability as a lipase substrate.

3.6.3. Enzyme regulation

Bacteria can use diverse carbon sources as catabolites. The enzymes for metabolizing these different substrates can be provided in two ways. A bacterium could constantly synthesize all of the enzymes or else could activate enzyme synthesis only as necessary to metabolize whatever particular catabolite happens to be present and/or is favorable from the energetic point of view. The latter case is additionally advantageous from an evolutionary perspective and is regulated by enzyme induction and repression.

The classic example of adaptation is the response of *Escherichia coli* cells to the presence of the disaccharaide lactose in its growth-medium as explained by the model of JACOB AND MONOD (1961). In this bacterium three proteins are involved in lactose metabolism: ß-galactoside permease, ß- galactosidase, ß-galactoside transaetylase. When *E. coli* is grown in a medium that does not contain a ß-galactoside, only low, uninduced levels of the three enzymes are present. When lactose or another ß-galactoside is added to the medium, the three proteins are induced and increase markedly (fig. 3.4).

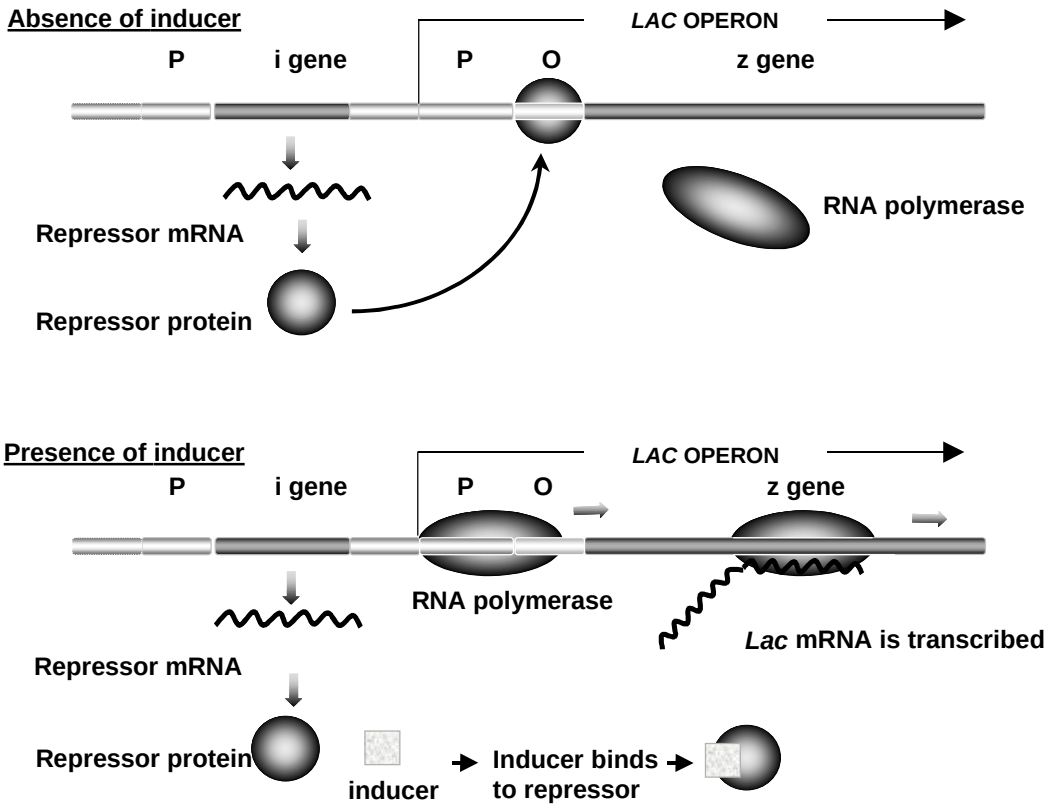


Fig. 3.4. Regulation of the lac operon according to the model of JACOB AND MONOD (1961).

Between the promoter, the site were polymerase binds, and the z genes, a region called the operator is located. The operator is a region were a regulatory protein, the *lac* repressor protein, binds (fig. 3.4). (A *lac* repressor is constitutively synthesized at very low levels in normal cells.) In absence of an inducer, the regulatory protein stays bound to the operator region and interferes with the binding of the polymerase to the promoter, preventing the transcription of the locus (repressing the transcription). When present, the inducer binds to the regulatory protein thereby undergoing a change in conformation and hence causes it to dissociate from the operator, thereby permitting transcription of the three *lac* genes to take place (transcription is derepressed).

An additional regulatory mechanism is carbon catabolite repression (CCR). The best understood example of CCR is the repression of metabolism of alternative sugars by glucose in the enteric bacteria *E. coli* and *Salmonella typhimurium*. This mechanism was first explained by the model of JACOB AND MONOD (1961) and has been the paradigm for carbon catabolite repression (CCR) in bacteria and gene regulation in general.

The *lac* promoter has two regions: 1) the region immediately adjacent to the *lac* operator were RNA polymerase binds; and 2) the CAP site, a binding site for the binding of the *catabolite activator protein* (CAP) (fig. 3.5).

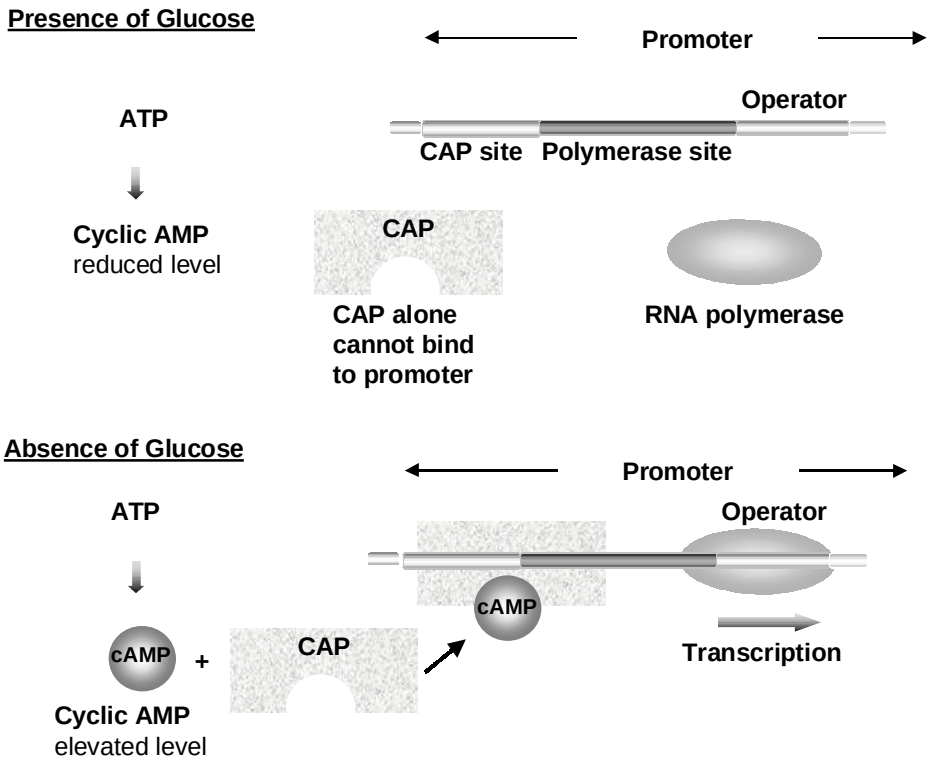


Fig. 3.5. Role of glucose in regulating the *lactose* operon by carbon catabolite repression in *E. coli* after JACOB AND MONOD, 1961.

When CAP is absent at this binding site, the polymerase cannot bind. Yet, CAP itself cannot bind to the *lac* promoter unless cAMP is bound to it. In the presence of glucose, cAMP levels are reduced so that CAP does not bind to the promoter and consequently the RNA polymerase does neither bind nor transcribe the lac genes, and vice versa. Hence, carbon catabolite repression (*CCR)* in E. coli is mediated by activation of transcription exerted by catabolite activator protein *(CAP)* complexed with cAMP which is present in elevated levels in response to the presence of the more favourable C-source (fig. 3.5). However, recent findings suggest additional mechanisms to be involved in Gram negative bacteria such as the “inducer exclusion” as reviewed by POSTMA ET AL. (1993) and STÜLKE AND HILLEN (1999).

The CCR mechanism in Gram negative and Gram positive bacteria (fig. 3.6) were found to be effected by the proteins of the phosphoenolpyruvate (PEP)-dependent sugar transporting phosphotransferase system (PTS), but the proteins that are directly involved in regulation and the mechanisms responsible for this control are completely different (as reviewed by HUECK AND HILLEN, 1995 and STÜLKE AND HILLEN, 1999). (The only common feature of CCR in *Escherichia coli* and *Bacillus subtilis*, for example, is that it is mediated at the level of transcription of target genes in both organisms).

CCR mechanisms of the group of Gram-positive bacteria are summarized and demonstrated in fig. 3.6.

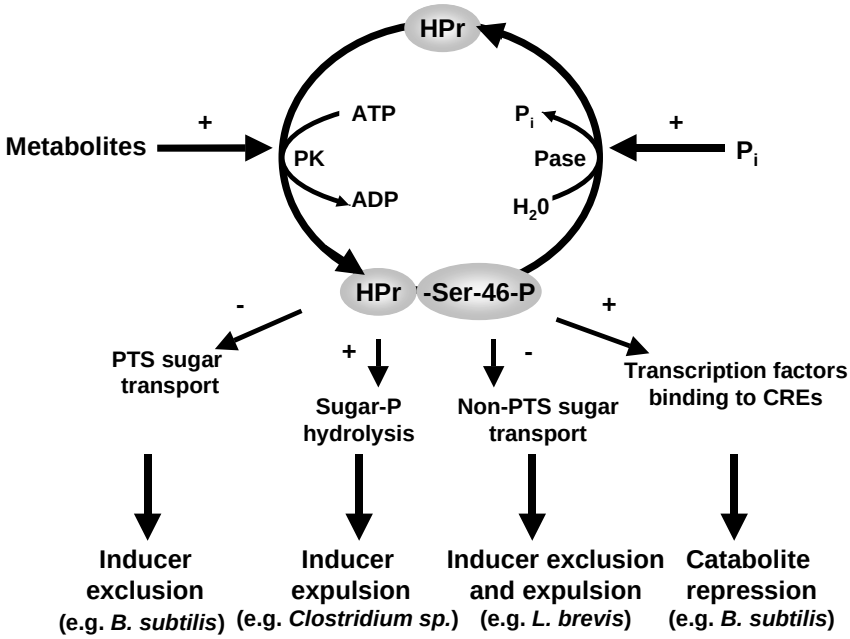


Fig. 3.6. Proposed function of the HPr(Ser)-phosphorylation by the ATP-dependent metabolite-activated HPr(ser) kinase in low-GC Gram-positive bacteria. Abbreviations: HPr, heat-stable phosphocarrier protein of the phosphotransferase system (PTS); PK, HPr(ser) kinase; Pase, HPr(ser-P) phosphatase; +, activation; -, inhibition; CRE, catabolite-responsive element (after SAIER ET AL., 1996).

Accordingly, inducer exclusion is effected by an inhibition of the PTS, while inducer expulsion is a result of the activation of a sugar-P phosphohydrolase which results in the export of the free sugar. Inducer exclusion and expulsion is a consequence of uncoupling H^+ symport from sugar transport via non-PTS transport system, while carbon catabolite repression occurs due to an enhanced binding of repressor proteins to the control regions of catabolite sensitive operons.

4. Results and Discussion

4.1. Assessment of anaerobic biodegradability of polyesters with anaerobic mixed microbial populations

With the aim of gaining more information about the anaerobic degradation of plastic materials it seemed to be sensible to clarify whether or not the chosen polyesters are susceptible on principle to anaerobic microbial attack. For these explorative tests, it is convenient to use mixed microbial populations to simulate different anaerobic microbial environments. Since different polyesters with different structures are studied, the microbial population should be as versatile as possible. Indeed, sludges and sediments are known to inhabit a broad spectrum of organisms.

Polyesters end up after their usage in either technically managed systems for controlled disposal or in natural environments as plastic litter. Therefore, the following different mixed microbial populations representing these two categories were chosen for these explorative experiments:

Technically managed and controlled disposal systems:

- Anaerobic sludge from a waste water treatment plant (**w**aste **w**ater **s**ludge: *WWS)*
- Anaerobic methane producing sludge from a laboratory reactor fed with waste water from the sugar industry (**l**aboratory **s**ludge: *LS)*
- **T**hermophilically treated **b**io**w**aste (*TBW*) from the anaerobic biowaste treatment plant in Watenbüttel, Germany.

Natural environment:

- **A**naerobic river **s**ediment *(AS)*

To simulate degradation under natural and practically relevant conditions, the polyesters were directly inserted into the native sludges and the sediment. The close contact of the polyester material and the microbial population inhabiting the sludges for the chosen period of incubation should allow and/or favor the natural enrichment of potential polyester depolymerizing anaerobes. These enrichment cultures served additionally as basis for the further screening for polyester degrading microorganisms.

4.1.1. Gravimetric monitoring of biodegradation

The present work concentrates on the mesophilic anaerobic break down of polyesters since most anaerobic environments and the majority of anaerobic waste treatment processes are mesophilic.

Polyester degradation is a multi-step-process initiated by:

1) the depolymerization of the polymer chain followed by
2) the dissolution of intermediates and
3) the subsequent metabolization and mineralization of the depolymerization products.

With the relatively simple method of weight loss determination, step 1 and step 2 of the subsequent degradation processes, leading to a disintegration of the polymer, can be screened. In many cases, the primary disintegration is supposed to be the rate limiting step, controlling the whole biological degradation. However, it has to be stressed that this preliminary gravimetric tests do not prove the final biodegradability in terms of mineralization. This will be checked in the later chapters of this work.

The natural poly(hydroxyalkanoates) PHB and PHBV were previously reported to be biodegradable with unidentified mixed microbial populations under anaerobic conditions and served in these investigations as positive reference materials. For the synthetic aliphatic polyester PCL literature provides different/contradictory results and to the author's knowledge no scientific reports about the anaerobic biodegradability of the other synthetic aliphatic polyester SP 4/6 and the synthetic aliphatic-aromatic polyester BTA 40:60 have been published up to now (for the chemical structures see Fig. 7.1).

Fig. 4.1. shows the mean weight loss data of three polyester films (surface area: 39.3 cm^2; m_0 = 39 – 49 mg) of each of the chosen materials incubated for 14 weeks at 35 °C in three different anaerobic mesophilic sludges.

Generally, all the materials exhibited at least some weight losses in the three anaerobic environments under mesophilic conditions (with the exception of SP 4/6 where no weight loss was determined in the anaerobic river sediment). However, there is a clear difference between the biologically induced disintegration detected for the different polyesters. The natural PHAs showed a high degree of disintegration ranging from > 60 % weight loss in the laboratory sludge to ≥ 100 % weight loss in the waste water sludge and the anaerobic river

sediment. Within the group of synthetic polyesters only PCL exhibited a moderate biodegradability. The maximum weight losses observed after the 14 weeks incubation period ranged between 25 % to 30 %. For the other synthetic polyesters (SP 4/6 and BTA 40:60) only in the laboratory sludge a significant weight loss (maximum of 2 mg) out of the experimental error could be observed. Since in all other tests the absolute differences in film weights was lower than 1 mg, conclusions about a biological attack cannot be drawn.

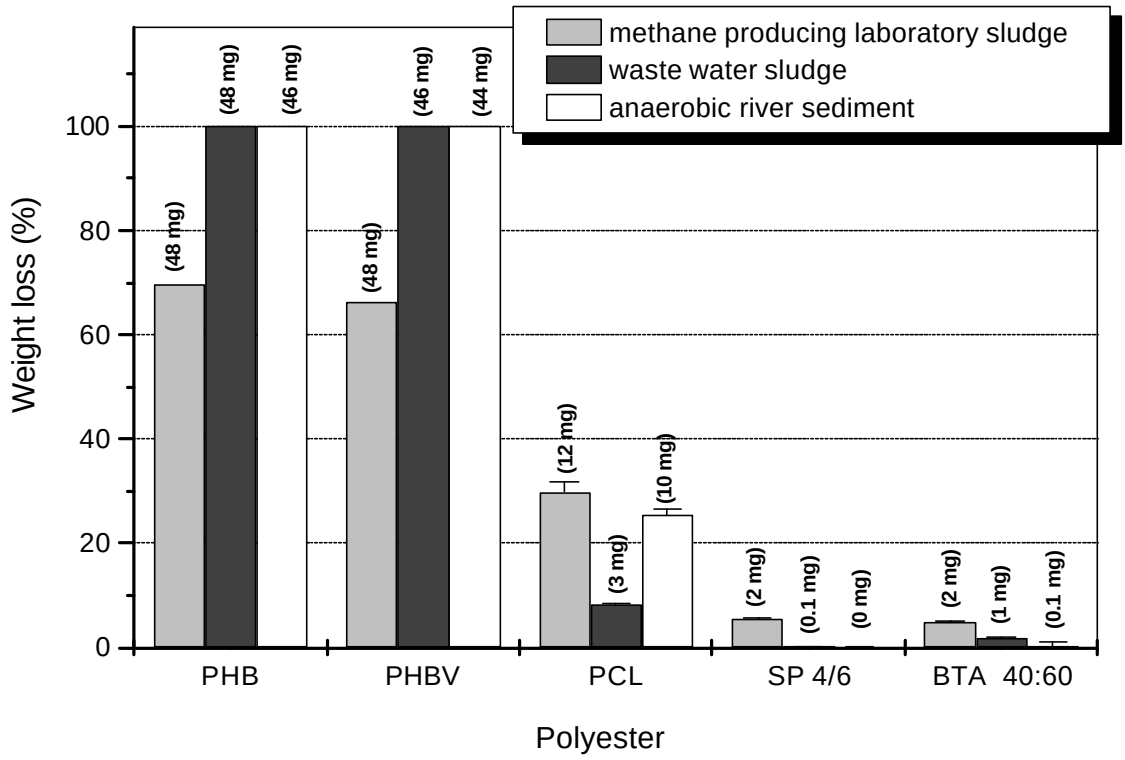


Fig. 4.1. Biological hydrolysis of the polyesters in different anaerobic environments after 14 weeks at 35 °C. (Polyester films: Ø = 25 mm; surface area: 39.3 cm^2; m_0 = 39 – 49 mg; n = 3 films per test.)

The question arose, if altering the sludge concentration would affect the anaerobic biodegradability of the polyesters under test. SHELTON AND TIEDJE (1984) stated that 10 % diluted sludges contain all necessary nutrients and fulfil the growth requirements of anaerobic microorganisms. Diluted and undiluted sludges, however, differ in the density of the microbial population on one hand, and on the concentration of nutrients - including the carbon sources - on the other hand. Diluting the sludge results in the dilution of the additional carbon source present in the sludge. It was questioned whether the lower carbon content of the sludges alters the anaerobic biodegradability of the polyesters. Therefore, the

influence of sludge concentration on the biodegradability of the polyesters under test exemplified by the laboratory sludge (comprising the highest weight losses for the synthetic polyesters) was examined.

Results shown in Fig. 4.2 a, b. show no significant differences in the weight loss data for the degradation in a diluted and undiluted sludge after incubation for seven weeks at 35 °C. Generally the same trend of biodegradability of the different polyesters as described in fig. 4.1 was obtained (PHB, PHBV > PCL > SP4/6, BTA 40:60) in both systems. Hence, the concentration of the sludge is negligible and further experiments were performed with undiluted sludges.

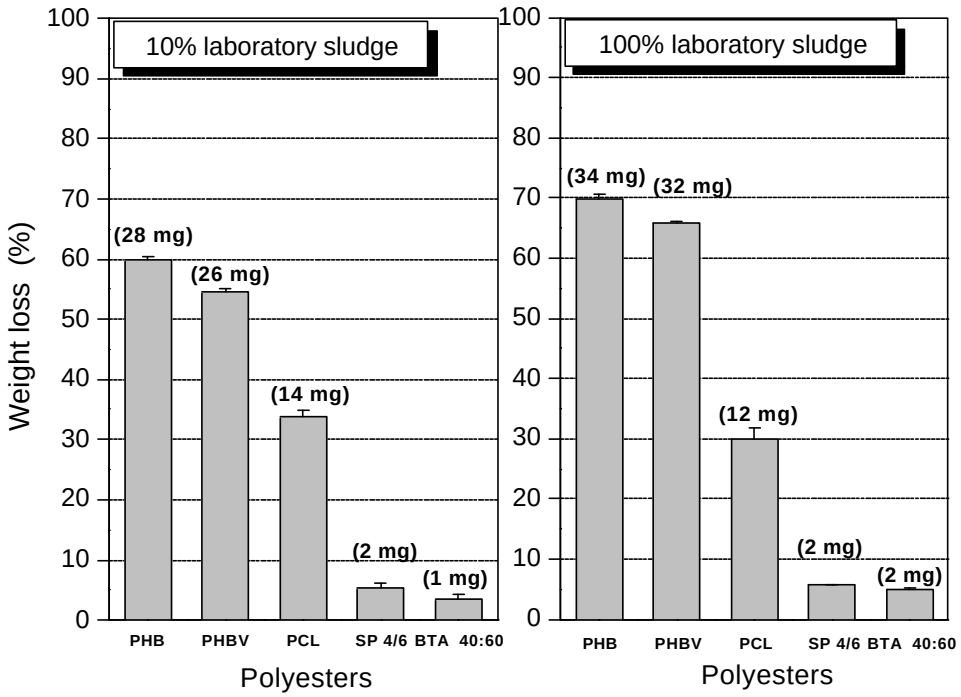


Fig. 4.2 a, b. Biological hydrolysis of different polyesters in diluted (a) and undiluted (b) laboratory sludge after 7 weeks at 35 °C. (Polyester films: Ø = 25mm; Surface area: 39.3 cm^2; m_0 = 39 – 49 mg; n = 3 films per test.)

Summarizing it can be stated, that for PHB and PHBV as well as PCL a definite anaerobic attack was shown within the incubation period of 7 or 14 weeks. The biologically induced weight loss of SP 4/6 and BTA 40:60 is, however, very small and lies just above the accuracy limit of the test method applied. At this point, the effect of the different sludges cannot be interpreted, since highest weight losses were achieved in the laboratory sludge for the synthetic but not the natural polyesters. On the contrary, natural polyesters lost more weight than the synthetic polyesters in the waste water sludge.

Disadvantageous is the fact, that the tests provide only one point measurements. Thus no detailed information about the degradation progress (lag phase, degradation phase) can be obtained and only average degradation rates can be estimated. PHB and PHBV films, for example, were already completely disintegrated at the sampling time in waste water sludge and the anaerobic river sediment (Fig. 4.1) after 14 weeks of incubation.

4.1.1.1. Thermophilic conditions

It was interesting to question, if it is possible to enhance the anaerobic biodegradability of especially the two polyesters with technical relevance (PCL and BTA 40:60) through an increase in incubation temperature. It must be pointed out here, that anaerobic and thermophilic waste treatment processes do exist. In addition, it has been observed, that increasing the degradation temperature may have a positive effect on biodegradability. This would be of special interest for a polyester like BTA 40:60, which exhibited a low biodegradability under mesophilic conditions (fig 4.1 and 4.2). The enhanced biodegradability of polyesters under aerobic thermophilic conditions was previously documented by KLEEBERG (1999) and interpreted by MARTEN (2000).

In the following investigations, an improved test with multiple-point measurements of weight loss was used. This test provides additionally information of the time course of the degradation and hence the possibility of a (course) calculation of degradation rates. In a comparative study (fig. 4.3 a, b) the effect of mesophilic versus thermophilic conditions on the biodegradability of the polyesters under investigation was determined. In this case the materials PCL 787 and BTA ZK 1094, two commercial products having higher melting points and molecular weights, were used to reduce abiotic hydrolysis expected to occur under thermophilic conditions.

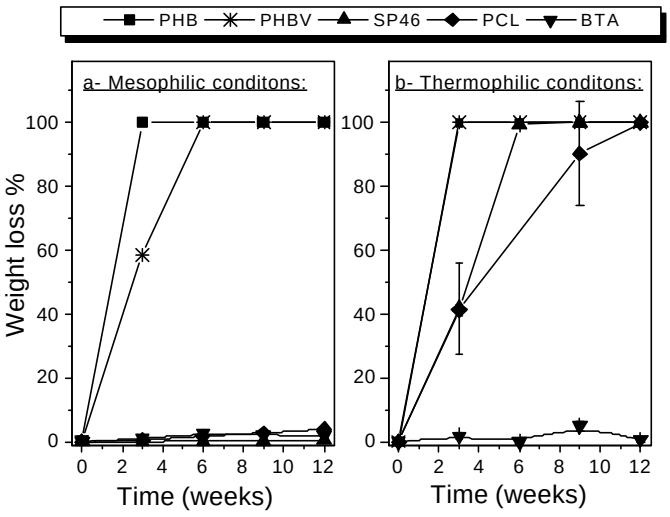


Fig. 4.3 a, b. Comparison of the time course of degradation of different polyesters under (a) mesophilic (*WWS*; 37 °C) and (b) thermophilic (*TBW*; 50 °C) conditions over a test period of 12 weeks. (Polyester films: Ø = 19 mm; surface area: 22.7 cm^2; $m_{0\ PHB, PHBV, BTA}$ = 60 - 76 mg; $m_{0\ PCL, SP\ 4/6}$ = 80 - 100 mg; n = 3 films per test).

For an easier comparison, fig. 4.4 compares the maximum degradation rates calculated from the linear part of the obtained degradation curve (fig. 4.3) ($\Delta m/\Delta t$) for the different polyester under mesophilic and thermophilic conditions. Under thermophilic conditions generally higher maximum calculated degradation rates were obtained as compared to mesophilic conditions. Even higher degradation rates must be anticipated for PHB and PHBV, since at the first sampling the material was already completely disintegrated. A 25 fold increase of biodegradability was determined for PCL (abiotic hydrolysis of this material did not exceed 1 %). For SP 4/6 even a 380 fold increase of the observed biodegradability (abiotic hydrolysis 0.2 mg ≈ 0.2 %) was obtained incubating the material at 50 °C. The apparent increase of 6.5 fold for BTA was, however, entirely due to abiotic hydrolysis (M_w and M_n were reduced to about 50 % after 3 weeks).

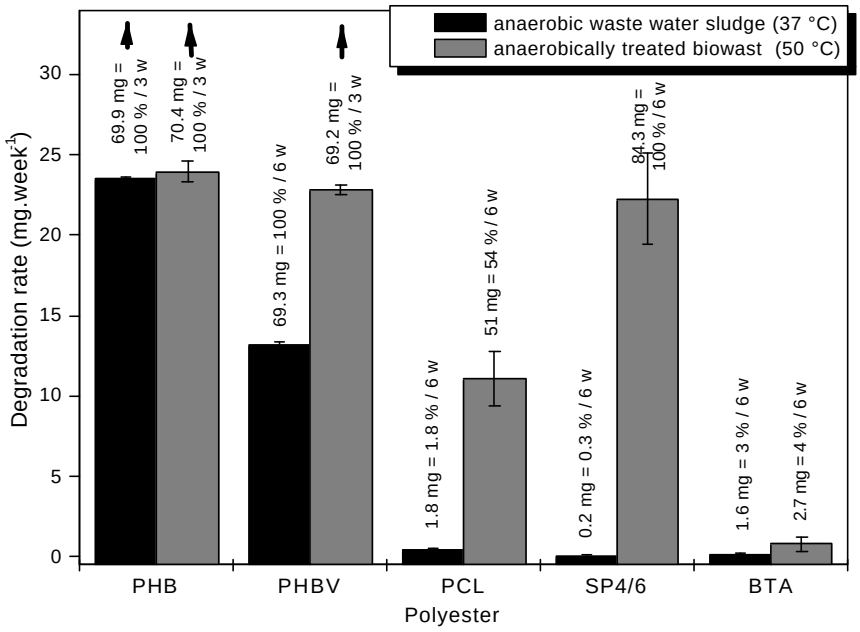


Fig. 4.4. Comparison of the maximum calculated degradation rates under mesophilic (*WWS*; 37 °C) and thermophilic conditions (*TBW*; 50 °C) over a test period of 12 weeks. (Polyester films: Ø = 19 mm; Surface area: 22,7 cm^2; $m_{0\ PHB,\ PHBV,\ BTA}$ = 60 - 76 mg; $m_{0\ PCL,\ SP\ 4/6}$ = 80 - 100 mg; n = 3 films per test).

Increasing the incubation temperature, therefore, is favorable considering practical aspects, if degradation velocities/rates are investigated, since the incubation time can be shortened. During the same incubation periods additional degradation potentials for the polyesters are explored, i.e. under thermophilic conditions the biodegradability of degradable polyesters such as PHBV and even PCL is increased. A polyester like SP 4/6, which mesophilicaly showed only minor weight losses, is rendered bio-available. It must be pointed out, that thermophilic sludges inhabit a different microflora with different biochemical potentials. On

the other hand, BTA remained persistent or inaccessible to microbial attack within the chosen time of incubation. It can thus be assumed, that BTA 40:60 is neither biodegradable under the chosen mesophilic nor thermophilic anaerobic conditions.

4.1.1.2. Influence of blending with starch

Alternatively to the effect of increasing the incubation temperature, the effect of blending the polyesters PCL 787 and BTA ZK 1094 with starch was studied. These materials comprise polyesters with technical relevance which are already on the market. Generally it was questioned, if blending such polyesters with starch, which is a readily metabolizable polymeric substrate for many anaerobic microorganisms, would increase the degradation rates of the blend as a whole. In addition, it was aimed to investigate if BTA, which showed only minor weight losses in the previous experiments, in form of a starch blend would be biodegradable or not.

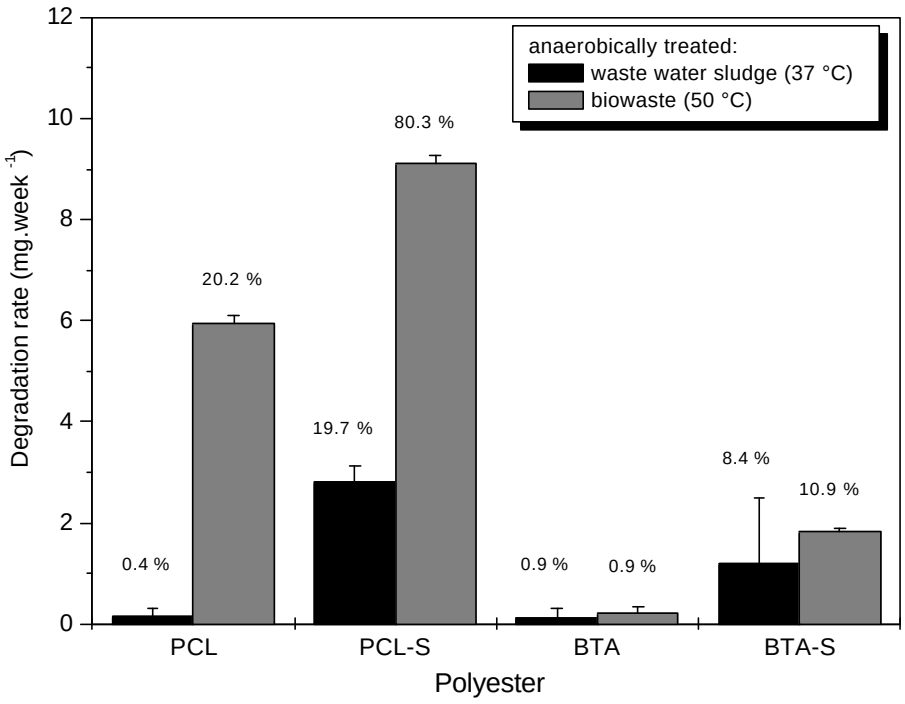


Fig. 4.5. Comparison of the maximum calculated degradation rates of PCL 787 and BTA ZK 1094 and their starch blends under mesophilic (*WWS*; 37 °C) and thermophilic conditions (*TBW*; 50 °C) over a test period of 12 weeks. Numbers represent absolute weight loss percentages. (Polyester films: Ø = 19 mm; Surface area: 22,7 cm^2; $m_{0\text{-}PCL}$ = 95 – 99 mg; $m_{0\text{-}PCL\text{-}S}$ = 31 - 34 mg; $m_{0\text{-}BTA}$ = 46 – 50 mg; $m_{0\text{-}BTA\text{-}S}$ = 60 – 65 mg n = 3 films per test).

Fig. 4.5. clearly shows that blending PCL 787 and BTA ZK 1094 with starch (starch content is 40 % and 32 %, respectively) increases weight losses. This effect is even more evident

under thermophilic conditions. Weight losses due to abiotic hydrolysis of PCL materials did not exceed 10 %, while BTA and BTA-S weight losses at 50 °C are entirely due to abiotic hydrolysis as clearly demonstrated by Fig. 4.6.

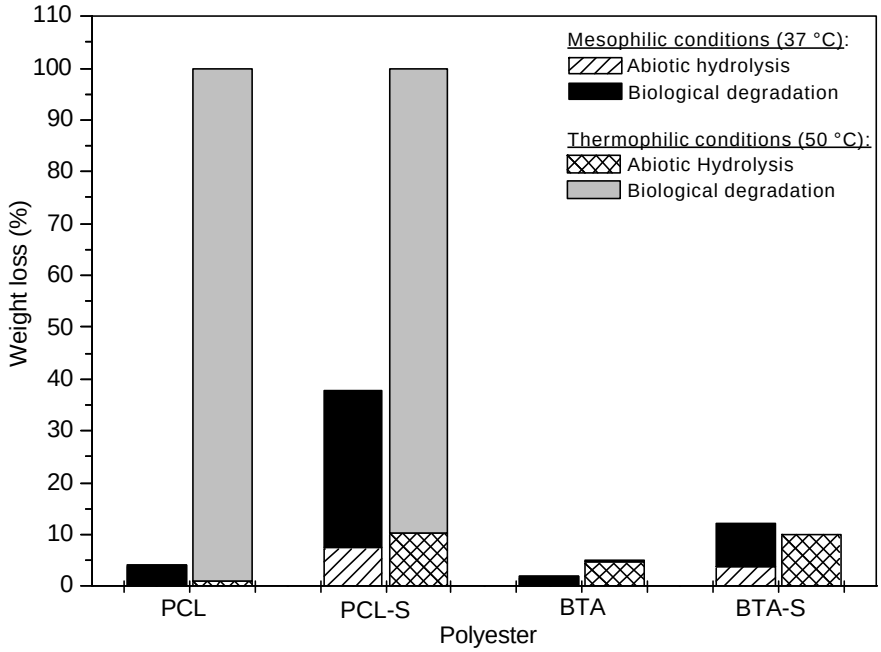


Fig. 4.6. Biotic and abiotic degradation of PCL, BTA and their starch blends under mesophilic (*WWS*; 37 °C) and thermophilic conditions (*TBW*; 50 °C) over a test period of 12 weeks. (Polyester films: Ø = 19 mm; Surface area: 22,7 cm^2; $m_{0\text{-}PCL}$ = 95 – 99 mg; $m_{0\text{-}PCL\text{-}S}$ = 31 - 34 mg; $m_{0\text{-}BTA}$ = 46 – 50 mg; $m_{0\text{-}BTA\text{-}S}$ = 60 – 65 mg; n = 3 films per test).

Consequently, the question arises if the increase in weight losses after blending the polyesters with starch are only caused by a selective removal of the well degradable starch fraction or if the starch addition also leads to an increased degradation of the polyester component.

At 50 °C no direct comparative investigations of the polyester films incubated under thermophilic conditions were possible since PCL-S film were completely disintegrated (100 % degradation) at the time of the first sampling and in case of BTA-S films weight losses were entirely due to abiotic hydrolysis as shown in Fig. 4.6. Thus the further discussion is based on results obtained at 37 °C and weight loss data at an intermediate degradation time (3 weeks) is regarded.

Fig. 4.7. shows that after incubation for three weeks at 37 °C in waste water sludge degradation of the starch blended materials PCL-S and BTA-S increased by 2 fold for PCL and 4 fold for BTA.

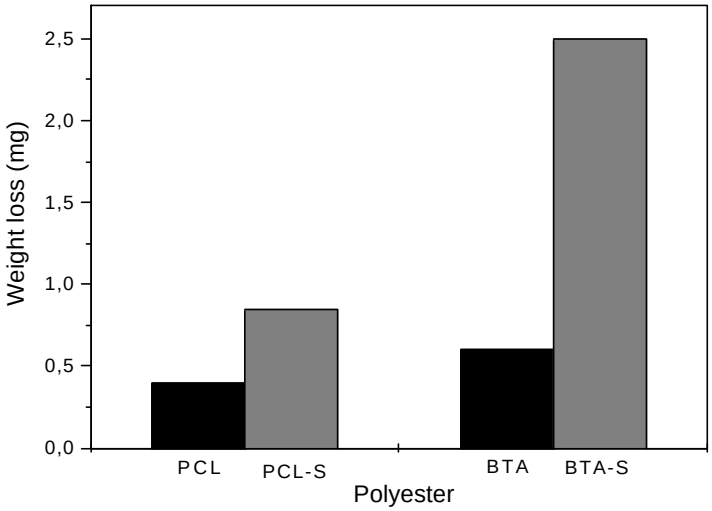


Fig. 4.7. Comparison of absolute weight losses of the polyester material in blended and non-blended PCL and BTA after 3 weeks under mesophilic conditions (*WWS*; 37 °C). (Polyester films: Ø = 19 mm; Surface area: 22,7 cm^2; $m_{0\text{-}PCL}$ = 95 – 99 mg; $m_{0\text{-}PCL\text{-}S}$ = 31 - 34 mg; $m_{0\text{-}BTA}$ = 46 – 50 mg; $m_{0\text{-}BTA\text{-}S}$ = 60 – 65 mg; n = 3 films per test).

From the material composition data before and after the degradation experiment (Fig. 4.8 a, b) it can be seen that the weight loss of PCL-S films was predominantly due to starch degradation. During the degradation, the starch content for the PCL blend changed from 39 % to 14.5 % and for the BTA blend from 31.2 % to 25.8 %. From that it can be calculated, that 24.7 % of the weight loss for PCL-S and 5.4 % of the weight loss for BTA-S was caused by a selective starch degradation.

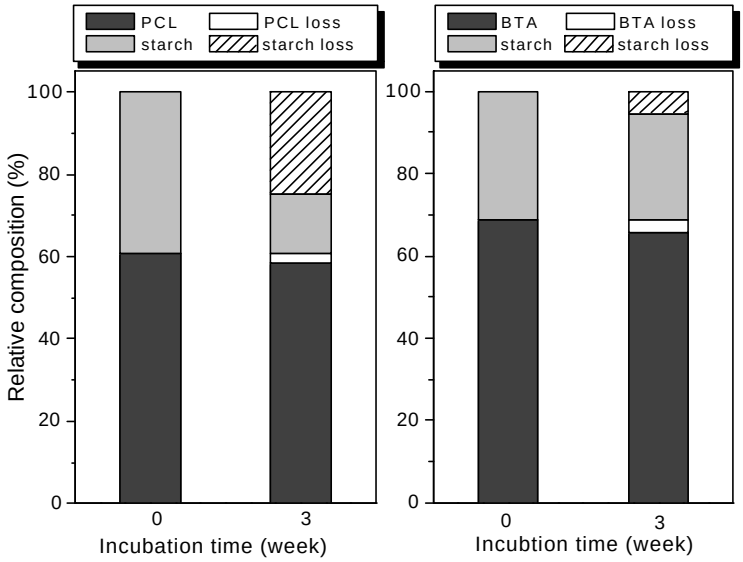


Fig. 4.8 a, b. Relative composition of (a) PCL-S films and (b) BTA-S films before and after incubation for 3 weeks at 37 °C in anaerobic waste water sludge. (Polyester films: Ø = 19 mm; Surface area: 22,7 cm^2; n = 3 films per test).

Generally, the overall increase in biodegradability of the blended polyesters may be due to one or several combined reasons as follows. The total degradation velocity can generally be

increased by, for example, increasing the available surface area given that starch is degraded faster than the polyester matrix. The loss of 24.7 % and 5.4 % starch out of the polyester-starch matrix of PCL-S and BTA-S, respectively, would consequently increase the porosity, i.e. the surface area available for enzymatic attack and hence increase the accessibility of the depolymerizing enzymes to the polyester material. Secondly, the enhancement of biodegradability may be due to the sum of the two subsequent processes, namely, the degradation of starch and the degradation of the polyester. Another possible consideration is, the improvement of the nutritional conditions for the involved microflora due to the presence of the readily degradable starch, which may also enhance biodegradation. Finally, the alteration of polyester properties such as variation of the cristallinity or of the melting temperature, affecting the biodegradability of the polyester component, may be considered.

Although first results about the principle anaerobic susceptibility of the polymers can be obtained from the weight loss measurements in the sludges, the test system exhibits several limitations. In case of material disintegration in the sludge or sediment a quantitative recovery is not always guaranteed. Due to the interference with and disturbance of the test system (introduction of oxygen) at the point of polyester film recovery, the test cannot be extended after sample recovery. Hence, each test vial delivers at the most one data point. In addition, weight loss data confirm solely the first and second stages of degradation, namely the depolymerization of the polymer chain followed by the dissolution of the depolymerization products. The metabolization of the depolymerization products by the mixed microbial populations in the sludge/sediment and the eventual mineralization, i.e. the “ultimate degradation” remains questionable.

To overcome the mentioned disadvantages, a second test system based on the determination of the overall produced biogas as an indicator of degradation was used for further investigations.

4.1.2. Determination of the produced biogas

Under anaerobic conditions, the degradation in terms of complete mineralization is a complex process which involves different kinds of microorganisms (see chapter 3.3. and fig. 3.1). The first stage in the degradation process is the breakdown of the polymer chains into smaller organic components by extracellular enzymes of the degradative microbial community. Fermentative bacteria take over to produce volatile fatty acids and esters along with CO_2 and H_2. Then, acetogenic bacteria convert the reduced fermentation products to

acetate. The methanogens thereafter, finally utilize acetic acid, CO_2 and H_2 to produce CH_4 and CO_2 (see chapter 3.3). The biogas produced, therefore, gives an indication of the total anaerobic activity occurring with each polymer system starting from depolymerization of the polymer chain followed by the metabolization of the depolymerization products by the mixed microbial populations and their eventual mineralization.

The method used is based on the volumetric measurements of the biogas and degradation is expressed in terms of percentage of theoretical gas production based on the stoichiometry of the mineralization to CH_4 and CO_2 based on the Buswell-equation (BUSWELL AND MÜLLER, 1952) (see chapter 7.5.2.). Using this test method the time dependent mineralization of different polyesters can easily be followed, thereby gaining detailed information about the degradation progress (lag-, exponential degradation-phase -, ...).

4.1.2.1. Degradation test with predigested native sludges

For these experiments the test set up with anaerobic sludges containing each one polyester film was thermostated in a chamber with a constant temperature of 37 °C. The percentage of degradation (%ThBiogas) was determined as the ratio of the cumulative net gas production to the theoretical value calculated from the C-content of the sample (see chapter 7.5.2.).

SHELTON AND TIEDJE (1984), assessing the biodegradability via biogas production found, that chemicals which are difficult to degrade only showed biogasification in concentrated sludges. Since a limited biodegradation of the synthetic polyesters was observed in the previous experiments (Fig. 4.1 – 4.8), first undiluted sludges were used for this experiment. In order to reduce the background gas evolution the sludges were predigested for a period of seven days or until no biogas production, caused by readily digestible organic materials, was recorded.

The degradability of the polyesters expressed as percentage of the theoretically maximum produced biogas (CH_4 and CO_2) by predigested laboratory and waste water sludge supplemented with the different polyesters (two parallel test set ups) at 37 °C as a function of exposure time over a period of 6 weeks is presented in Fig. 4.9 a and b.

Determining the biogas production, on principle the same trend of biodegradability of the polyesters under test previously observed by weight loss measurements was observed. PHB was completely mineralized within 8 to 18 days, while PHBV clearly showed a slower mineralization and degradation than the homopolyester. Also, PCL showed a definite

mineralization above the accuracy limit of the applied test method. On the other hand, SP 4/6 and BTA 40:60 showed only low mineralization after 42 days of incubation, as compared to the relatively high background gas values (33 - 37 % of the determined biogas due to mineralization of the polyesters).

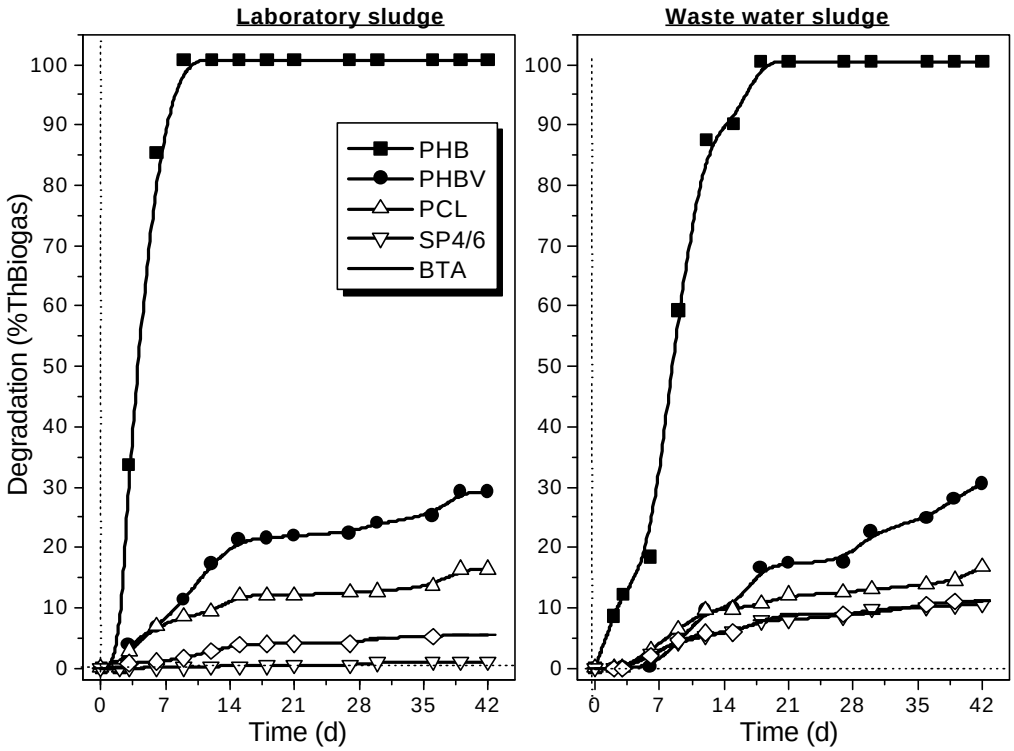


Fig. 4.9 a, b. Time dependent mineralization expressed as percentage of the theoretical biogas volume evolved from anaerobic laboratory and waste water sludge at 37 °C over a period of 42 days. (Polyester films: Ø = 19 mm; surface area: 22.7 cm^2; film $weights_i$: 35 – 40 mg; n = 2 films per test).

Determining the weight losses of the films at the end of the degradation test (table 4.1), it became obvious that PHBV, and PCL were faster depolymerized (weight loss 57 – 63 % and 30 %, respectively) than mineralized. This points to a temporary accumulation of the depolymerization intermediates. Similarly, REISCHWITZ AND COWORKERS. (1998) detected the accumulation of the intermediate hydrolysis products acetate, propionate, n-butyrate, iso-butyrate and n-valerate during PHBV mineralization using anaerobic sludge cultures as well as a selective sludge culture inoculum. They suggested an inhibition of acetogenic and methanogenic bacteria by the formed organic acids due to an imbalance between the high substrate content to the low bacterial mass, especially with the latter test system. In addition, they proved the accumulation of four different dimeric esters of 3-hydroxybutyrate and 3-hydroxyvalerate during the degradation of PHBV in an anaerobic sludge.

However, for SP 4/6 and BTA 40:60 lower weight losses compared to the biogasification data were determined in the waste water sludge (table 4.1). This points to an apparently higher biogas detection obviously due to the background gas evolution. Additionally, a lag phase of 2 to 3 days was observed in the waste water sludge, pointing to a required adaptation phase.

Table 4.1. Comparison between the experimentally obtained percentage of degradation as measured by biogasification and weight loss data of the polyester films.

Polyester	Laboratory sludge		Sewage sludge	
	% Biogas	% Weight loss	% Biogas	% Weight loss
PHB	100.82	100	100.54	100
PHBV	29.22	57	30.57	63
PCL	16.40	30	16.70	29,8
SP 4/6	1.08	1.2	10.59	2.1
BTA 40:60	5.50	0.5	11.13	1

4.1.2.2. Influence of sludge dilution

To reduce the background gas evolution, which turned out to be a limiting factor in assessing biodegradability of the aliphatic polyesters, a mineral salt medium inoculated with an enrichment culture inoculum (10 % (v/v)) from a laboratory sludge (supplemented with the five different polyesters under test for a period of 18 weeks, at 37 °C) was used. The polyesters SP 4/6 and BTA 40:60 having a limited biodegradability were omitted in this test and SP 3/6 known to be more easily biodegradable under aerobic conditions (WITT ET AL. 1997) was used instead (Fig. 4.10).

Compared to the biogasification in the concentrated laboratory sludge, a clear lag phase of about 5 days is observed in the inoculated mineral medium. Additionally, the diluted system seems to be more sensitive as the pronounced stepwise degradation of the polyesters points to population dynamics through adaptation phases of the different bacterial trophic groups (fermentative bacteria, acetogenic bacteria, methanogenic bacteria). This may be explained by the lower density of microbial cells, and a lower buffer- as well as adsorption capacity of the highly diluted sludge. The effect of accumulating depolymerization and/or degradation products is therefore intensified and may adversely affect the highly sensitive methanogenic population.

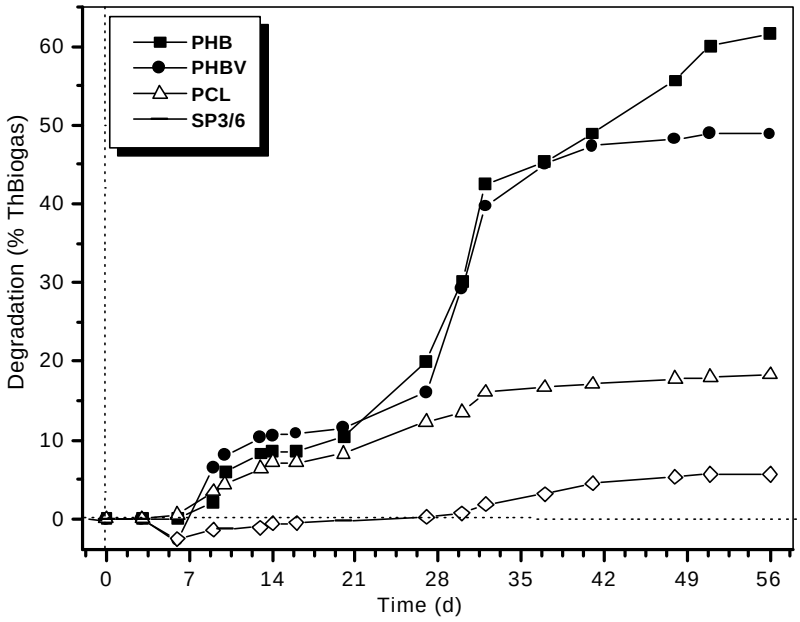


Fig. 4.10. Time dependent mineralization expressed as percentage of the theoretical biogas volume evolved from a MSV-medium inoculated with a laboratory sludge enrichment culture inoculum (10 % v/v) at 37 °C over a test period of 56 days.

Generally, no clear dependence of the degradation rates upon the initial microbial concentration can be stated. While PCL degradation is similar in both systems (approx. 16 – 17 % within 42 days), PHB mineralization is significantly slower in the mineral medium. In contrast PHBV mineralizes faster in the diluted system and reached almost the same degree of mineralization as PHB at the end of the test. However, the relative trend of the degradation rates of the different polyesters seems comparable to that observed in the concentrated sludges. Although SP 3/6 is reported to be more rapidly degraded than SP 4/6 under aerobic conditions, only a very low biogas development was observed for SP 3/6 under anaerobic conditions, still leaving open if these kinds of polyesters are really significantly attacked by anaerobic microorganisms.

The background gas evolution with this test was significantly lower (3 - 5 % of the determined biogas due to mineralization of the polyesters in the diluted system, compared to over 30 % in the native sludges) and hence no interference with the degradation data is to be expected. A higher degree of accuracy of mineralization data in the diluted system is therefore obtained. However, as mentioned before, the general trend of biodegradability of the different polyesters remained unchanged. Additionally, the average degradation rate for PHB in the diluted system is slower (compared to the one measured in the concentrated sludges, and is obviously due to the higher sensitivity of the involved microorganisms, as is shown by the stepwise degradation.

4.1.3. Discussion

Plastics occur as litter in anaerobic sediments, in landfills, were anaerobic conditions exist, or are incorporated in anaerobic waste treatment processes. Yet, little if any information is available about the fate of plastics such as polyesters in these environments. Thus, a main goal of the present work was to investigate the principal susceptibility of the different polyesters to anaerobic microbial attack. The main question to be answered was, therefore, whether the polyesters under investigation were generally susceptible to anaerobic microbial attack and to what extent anaerobic biodegradability can be expected in different anaerobic habitats such as technically managed systems and a natural anaerobic environment.

The natural polyesters PHB and PHBV were previously reported to be aerobically biodegradable in anaerobic sediments by sulphate reducing anaerobic bacteria (GUERRERO AND MAS-CASTELÀ, 1994; MAS-CASTELÀ ET AL., 1995), with a methanogenic coculture as well as sludges (BUDWILL ET AL., 1992; REISCHWITZ ET AL., 1998), and under simulated landfill conditions (MCCARTIN ET AL., 1990; SHIN ET AL., 1997). Hence, these polyesters were used as positive reference materials in this work. PÜCHNER, (1995) as well as FINK AND SCHÄFER (1996) reported the commercially interesting synthetic polyester PCL to be anaerobically resistant, using test conditions which are comparable to the first screening degradation tests used in the present work. Yet, the existence of PCL depolymerizing anaerobes was confirmed by a study of NISHIDA AND TOKIWA (1994A). On the other hand, no scientific reports about the anaerobic biodegradability for the synthetic polyesters SP 3/6, SP 4/6 or BTA 40:60 have been published to the author's knowledge, although the production of a copolyester comparable to the latter has been started on a several thousand tons per year scale (Ecoflex®, BASF-AG, Germany).

The first test system applied in this study depends on gravimetric monitoring of biodegradability. Weight loss of the polymer material incorporated in soils/sludges/sediments, thereby simulating the environmental conditions of interest, is the most widely and simplest test method used for monitoring polymer changes (AUGUSTA ET AL., 1992; MERGAERT ET AL., 1993; MAS-CASTELLÀ ET AL. 1995; WITT ET AL., 1995). Interpreting weight loss data in sense of depolymerization of the polyester material represents a simple and easy test method which delivers repeatable results for preliminary degradation studies.

Although the weight loss determination of polyester materials in sludges and sediments allows the principal evaluation of susceptibility of the materials under test, weight loss data

confirm solely the first stage of degradation namely the depolymerization of the polymer chain followed by the dissolution of the depolymerization products. The metabolization of the depolymerization products by the mixed microbial populations in the sludge/sediment and the eventual mineralization remains questionable.

A more advanced test system is, therefore, the determination of the produced biogas as a result of anaerobic biodegradation of polyesters. It is an indicator of the *ultimate biodegradability* (WAGNER, 1988, BAUMANN AND SCHEFER, 1990; MARTEN AND KELLER, 1991), i.e. the complete degradation and mineralization to CO_2, CH_4, H_2O and minerals. The amount of biogas produced as a result of polyester mineralization is stochiometrically calculated on the basis of the Buswell-equation after BUSWELL AND MÜLLER (1952).

Advantageous is the gained information about the time dependent mineralization and about the degradation progress (lag-, exponential degradation-phase -, ...) and the possibility of calculating degradation rates. Drawbacks of this system are the high background gas evolution rates, when concentrated sludges are used. These high background gas values may falsify the mineralization results, especially with polyesters having a limited biodegradability, e.g., SP 4/6 and BTA 40:60. On the contrary, if diluted sludges are used, a general sensitivity of the test system is observed and expressed in fluctuating/stepwise degradation which is dependent on the sludge composition, the physiological conditions/activity of the involved organisms, and the interaction of different microbial groups. The degradation behavior clearly demonstrates population dynamics. The inhibition of any one of the individual stages catalyzed by different trophic groups will have consequent adverse effects on other stages of the overall coordinated process of methanogenesis, i.e. the system is dependent on the efficiency and frequency of highly specialized and sensitive organisms (BRAUN, 1982).

Generally, the same trend of biodegradability of the different polyesters was observed for the polyesters under test independently of the applied test system, the type of sludge or its concentration: PHB, PHBV > PCL > SP 3/6, SP4/6, BTA 40:60.

This implies the impact of polyester properties rather than the nature of the sludge or sediment. In addition, the reproducibility with parallel tests is acceptable but low/unacceptable with subsequent tests. It must be anticipated that the sludge composition changes during storage even at low temperatures (4 °C).

Surprisingly, the anaerobic biodegradability of the synthetic polyesters with the exception of PCL seemed to be rather limited, yet the same materials are reported to be aerobically easily biodegradable (WITT ET AL. 1997; MARTEN, 2000). Therefore, the influence of elevating the temperature on the biodegradability of the synthetic polyesters was tested. Although the thermophilic conditions (50 °C) enhanced the anaerobic biodegradability of PCL and SP 4/6 significantly, BTA 40:60 remained persistent.

The biodegradability enhancement under thermophilic conditions is explained by the involvement of different organisms and an increase of polyester flexibility with increasing incubation temperature. Increase of temperature has a significant influence on the bioavailability and solubility of organic compounds. The elevation of temperature is accompanied by a decrease in viscosity and an increase in diffusion coefficient of organic compounds. Consequently, higher degradation rates due to smaller boundary layers are expected. At elevated temperature the solubility of polymeric compounds with a limited solubility such as starch, cellulose, proteins and polymers is drastically increased, allowing efficient bioconversion reactions due to high substrate concentrations (MÜLLER ET AL., 1998). Similar effects are expected for polyesters.

Marten (2000) showed that the difference between melting temperature of the polyester material and the incubation temperature is non-linearly proportional with the enzymatic degradation velocities. If this temperature difference is smaller than 30 °C (e.g. PCL, SP 4/6) a significant increase in degradation velocities is measurable. BTA 40:60, however, has a melting temperature of 180 °C.

Yet, our interest of the mesophilic anaerobic conditions outweighed the thermophilic biodegradation since most ecosystems, such as anoxic river and lake sediments (NEDWELL, 1984), the rumen of cattle or most anaerobic digesters (SIXT, 1982), subsurface soils and landfilling (SENIOR AND BALBA, 1987) are mesophilic. Thus, the biodegradation under thermophilic conditions was not investigated further.

Blending of BTA 40:60 with starch also did not result in the expected increase in the anaerobic biodegradability of this copolyester, further pointing to its resistance to anaerobic biodegradation, at least under the degradation conditions chosen for the present work.

In terms of biodegradation studies, the main problem is that in many cases the biological environments are poorly defined, and the small-scale laboratory test systems used to

measure biodegradability fail to simulate the “real” environmental conditions (DAY ET AL. 1994). As a solution to this problem the development of a test system with characterized single strains under controlled and identified cultural conditions is proposed.

4.2. Evaluation of anaerobic biodegradability of polyesters with pure single strain cultures

For all the tested polyesters at least some degree of biodegradability was observed in the test systems, applying the different anaerobic sludges (see chapter 4.1.1 and 4.1.2). These tests, however, are based on unidentified mixed microbial populations and the test environment is highly complex. Although guaranteeing the universality of the test system for different polyesters, it shows limits with respect to the accuracy of the test results. Additionally, this complexity of natural (anaerobic) environments renders defined mechanistic investigations of polymer degradation almost impossible.

The depolymerization of the (water insoluble) polymers to soluble oligomers and monomers by the attack of special extracellular hydrolyzing enzymes is the first step in the succession of the entire mineralization procedure. Thus, it was envisaged in the following to isolate from the previously described mesophilic sludges single strains of microorganisms, able to attack the polymer chains. This screening would allow on the one hand, to investigate the diversity of potential polyester degraders in the different anaerobic environments, and to clarify whether or not a single anaerobic strain is capable of polyester disintegration and subsequent metabolization on the other hand. Beside performing the comparative degradation studies under defined, controlled and optimized cultural conditions, it becomes possible to overcome fluctuations in degradation due to sludge compositions. It was intended to use these isolates in defined and improved laboratory degradation tests as a tool for mechanistic studies. Furthermore, first conclusions are expected on the degradation mechanism knowing which kind of microorganisms (and their corresponding extracellular enzymes) are responsible for the primary attack of the different polyester structures.

4.2.1. Development of a screening and isolation procedure

The degradation results previously obtained in chapter 4.1 ascertain, on principle, the presence of microorganisms capable of polyester depolymerization. Before being able to isolate these target organisms from the enriched consortia in the sludges, it was first necessary to develop an adequate screening procedure, especially adapted to the polymeric substrates and anaerobic conditions.

4.2.1.1. Development of polyester incorporation/emulsification method for media preparation

The most widely used screening method for polyester depolymerizing organisms is the so called “clear zone” method (AUGUSTA ET AL., 1993; JENDROSSEK ET AL., 1993B). The extracellular depolymerizing enzymes secreted by the target organism hydrolyzes the suspended polyesters in the turbid agar medium into water soluble products thereby producing zones of clearance around the colony. The main advantage of this test is that it is generally fast and simple, and allows the simultaneous performance of a great number of parallel tests.

Depending on the different physical properties of the used polyesters, the development of special emulsification methods for the different polyesters was necessary. For PHB and PHBV (see chapter 7.2.3.a) no special treatment was required and the polyester powder (particle size around 1 µm) was directly mixed into the minimal agar medium prior to sterilization.

The synthetic polyesters on the other hand, probably due to their low melting points and hydrophobic surfaces, agglomerated in the media. Therefore the direct incorporation of polyester powders into the liquid medium was not possible. Casting of the polyesters dissolved in an adequate solvent on the surface of the agar plates resulted in turbid plates but prevented growth of organisms. Probably the hydrophobic surface interrupted the nutritional transfer.

Hence, a polyester incorporation method preventing the agglomeration of the synthetic polyesters in liquid media had to be developed. A suitable procedure for preparing the agar containing liquid media could be established. The polyesters were first dissolved in di-chloromethane. The organic solution (5 % (w v^{-1})) was then emulsified by ultrasonication in a liquid and mineral salt media (see 7.2.3.b) containing agar powder (1.5 % (w v^{-1})). Afterwards, the emulsion was boiled under constant stirring for at least 30 minutes to completely evaporate the solvent. Once the characteristic color of resazurine had changed from pink to colorless, the dissolved oxygen as well as the solvent had been completely driven out of the medium. After sterilization by autoclaving, homogeneously turbid plates were obtained.

4.2.1.2. Roll tube method for initial screening and evaluation

For this method anaerobic "Hungate tubes" filled with 3 ml of the mineral agar medium containing the suspended PHB or PHBV or optionally the emulsified synthetic polyesters were autoclaved. Prior to solidification the tubes were rolled on a cold surface resulting in a thin film (approx. 2 mm) of agar medium on the walls of the "Hungate tubes". These tubes comprised a fast and simple tool to determine whether or not polyester depolymerizing microbes had been successfully enriched (see chapter 7.3.). The appearance of clear zones (Fig. 4.11) using the above described media proves that the colony in the center of the clear zone hydrolyses the polyester in the mineral agar medium.

Fig. 4.11. Clear zone formation in PHB-RAMM (see chapter 7.2.2. table 7.3) roll-tubes after 7 days at 37 °C (inoculated from a 14 weeks old laboratory sludge-PHB enrichment culture; chapter 7.6.1.).

The thin agar layer on the wall of the tubes ensures fast results (clear zones) and strictly anaerobic conditions are easily guaranteed in this tube–based clear zone method. However, some problems arose with the roll tube method, when using it for the isolation procedure of microorganisms.

The narrow necks of the tubes rendered isolation and subculturing of the tiny colonies very difficult and unpractical. Performing the screening experiments on plates (Fig. 4.12) instead of roll-tubes did not solve the problem since colonies were too minute for subsequent subculturing and isolation. This can be explained in part by the comparably long generation times and the low energy yields known for anaerobic organisms.

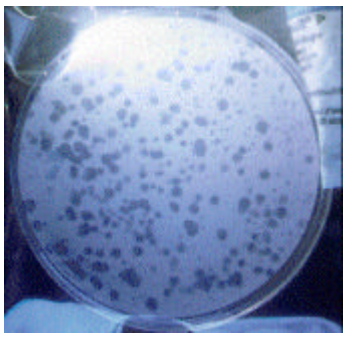


Fig. 4.12. Clear zone formation on PHB-RAMM plates (see chapter 7.2.2. table 7.3) after 7 days at 35 °C (inoculated from a 14 weeks old laboratory sludge enrichment culture containing PHB; chapter 7.6.1.).

4.2.1.3. Combining replica plating and clear zone formation for the isolation of polyester degrading anaerobes

To overcome the problem of low biomass formation (minute colonies) in the roll tubes and on the mineral agar plates, respectively, the screening and isolation method was modified. The isolation procedure consisted of at least four subsequent steps (see chapter 7.6.):

1) The enriched microbial population containing the potential polyester depolymerizing organisms was cultivated on rich complex media (see 7.2.2, table 7.3) leading to high cell densities, and large as well as cultivable colonies.

2) Then, morphologically different colonies were subcultured on polyester containing mineral salt agar plates and potential depolymerizing anaerobes were selected via clear zone formation.

3) In an additional step, the replica plating technique was applied (see 7.6.3) to clear zone forming colonies. The individual strains were tested for their ability to grow on and depolymerize the polyesters incorporated in the mineral-salt-vitamin-(MSV) agar plates supplemented with and without different co-substrates. The selection criterion was the ability to form clear zones.

4) Positive strains were isolated by picking the colonies using sterile tooth picks, further purified on complex media (see 7.2.2., table 7.3) using the standard spatial streaking method on solid agar media plates, and preserved on rich complex media. Eventually, the selected anaerobes were classified according to their degradation potential. Additionally, their growth requirements (different media, pH, temperature, supplements) were identified to allow the further optimization of their degradation capabilities.

4.2.2. Individual strains degrading the natural hydroxyalkanoates: PHB and PHBV

From enrichment cultures with PHB and parallel with PHBV incubated for 14 weeks at 35 °C in the different anaerobic sludges a total of 76 morphologically different anaerobic bacterial isolates were obtained on complex microbial media. These strains were tested for their ability to depolymerize PHB via clear zone formation. Among these 76 isolates, 12 strains formed clear zones on PHB-MSV mineral salt agar without any supplementation with cosubstrates. Further 18 isolates required the presence of an additional carbon source such

as acetate, crotonate or citrate for PHB depolymerization; glucose only supported the PHB depolymerization of one strain. These cosubstrates were chosen because they are readily metabolizable C-sources for many anaerobic microorganisms. Especially, acetate is required as a carbon source for the growth of heterotrophic anaerobic BACTERIA (TANAKA, 1995) and is known to act as a building block for biosynthetic purposes or as an electron acceptor (e.g., for *C. kluveri* and acetogenic anaerobes) (ANDREESEN ET AL., 1989) and BADER ET AL. (1980) reported crotonate to be an intermediate of 3-hydroxybutyrate (the monomer of PHB) fermentation for clostridia.

Table 4.2 lists the total number, isolation source and PHB depolymerizing potential expressed in clear zone diameter of the organisms isolated from enrichment cultures containing PHB and optionally PHBV as enrichment substrate.

Table 4.2 a and b. Screening of PHB-degrading organisms from different enrichment cultures with PHB or PHBV as an enrichment substrate

Microbial source	PHB as enrichment substrate					
	No. of organisms screened on PHB-medium supplemented with[a)]:					
	Total	no suppl.	+ Acetate	+ Crotonate	+ Citrate	+ Glucose
Laboratory sludge (LS)	**13**	6 (5-11mm)[a]	11 (5-18mm)	8 (5-26mm)	6 (17-32mm)	1 (5mm)
Waste water sludge (WWS)	**3**	2 (5-22mm)	1 (12mm)	3 (5-18mm)	0	0
Anaerobic river sediment (AS)	**3**	0	1 (6mm)	2 (4-12mm)	0	0
Total	**19**	**8**	**14**	**13**	**6**	**1**

Microbial source	PHBV as enrichment substrate					
	No. of organisms screened on PHBV-medium supplemented with[a)]:					
	Total	no suppl.	+ Acetate	+ Crotonate	+ Citrate	+ Glucose
Laboratory sludge (LS)	**4**	1 (5mm)[a]	4 (4-20mm)	1 (20mm)	1 (12mm)	0
Waste water sludge (WWS)	**6**	3 (2-8mm)	6 (8-24mm)	3 (18-24mm)	4 (12-30mm)	0
Anaerobic river sediment (AS)	**1**	1 (4mm)	0	0	0	0
Total	**11**	**5**	**10**	**4**	**5**	**0**

a) Numbers in brackets: diameter of clear zone formed on PHB or PHBV supplemented mineral salt agar plates after incubation for four weeks at 35 °C.

As expected from the weight loss experiments described above (see chapter 4.1.1.), all three different microbial sources are inhabited by PHB depolymerizing organisms. However, sludges from technically controlled processes (***LS*** and ***WWS***) harbor a broader spectrum of PHB–degraders (total of 26 strains) compared to the natural habitat (***AS***), from which only a total of 4 different PHB degrading isolates were obtained.

Interesting was the finding that more strains were isolated from the PHB enrichments (19 strains) than from the PHBV enrichment cultures (11 strains) although the microbial sources were identical. This fact correlates with the differences in the biodegradability of PHB and PHBV previously observed in chapter 4.1. It additionally points to the impact of polyester characteristics on the biodegradability of the polyester rather than the microbial population.

The 30 isolates can be divided into 11 subgroups depending on the different co-substrates supporting PHB-depolymerization. These 11 groups and their co-substrate spectra are summarized in Table 4.3 and the degradation behavior of one representative of each group is graphically illustrated in Fig. 4.13. The presence of at least 11 different groups of PHB degrading isolates points to the metabolic versatility of the anaerobic PHB-degrading bacterial population.

Table 4.3. Groups of different organisms depolymerizing PHB in absence or presence of different co-substrates.

No. of strains forming clear zones	PHB-MSV medium supplemented with				
	No suppl.	Acetate	Crotonate	Citrate	Glucose
1	+	+	+	+	+
4	+	+	+	+	-
4	+	+	+	-	-
1	+	+	-	+	-
2	+	-	+	-	-
2	-	+	+	+	-
1	-	+	+	-	-
2	-	+	-	+	-
9	-	+	-	-	-
3	-	-	+	-	-
1	-	-	-	+	-
Total: 30	**12**	**24**	**17**	**11**	**1**
100 %	**40 %**	**80 %**	**57 %**	**37 %**	**3 %**

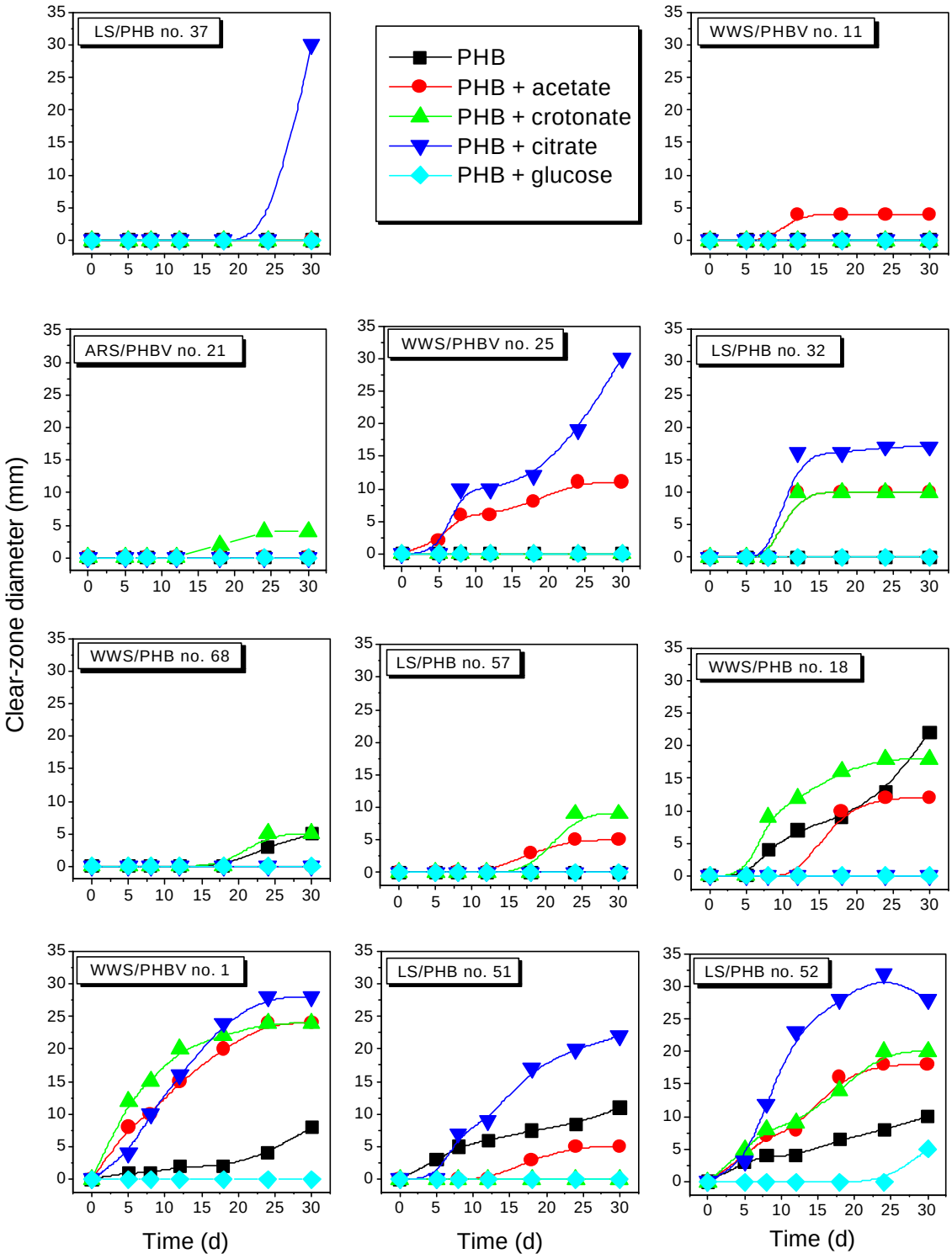


Fig. 4.13. Examples of different organisms and their PHB-degrading potential (clear zone diameter in mm) in presence of different co-substrates. (Description in boxes represent isolation source and enrichment substrate: e.g. LS/PHB no. 37 = strain number 37 isolated from a laboratory sludge enrichment culture containing PHB as substrate).

Generally, the addition of an additional carbon substrate (additional energy source) such as acetate, crotonate or citrate obviously enhanced biomass formation and hence clear zone formation. PHB depolymerization is mainly stimulated by acetate (80% of the total number of isolates) and crotonate (57 % of the 30 isolates), a fact which can be explained by acetate being an intermediary metabolite of many anaerobes (ANDREESEN ET AL., 1989) and crotonate is a key metabolite in 3-hydroxybutyrate metabolism (BADER ET AL., 1980).

Glucose on the other hand supported growth of the isolates but not clear zone formation with one exception. Obviously, glucose suppresses the PHB depolymerizing enzyme secretion, probably by catabolite repression (ANDREESEN ET AL., 1989; MITCHELL, 1998). Generally, isolates depolymerizing PHB (Fig. 4.13) in presence of acetate, crotonate or citrate or even glucose (e.g. strain no. 1, 51 or 52) exhibit a higher depolymerization potential than those which are more restricted, i.e. which depolymerize PHB only in presence of one or two particular cosubstrates (e.g. strain no. 68, 57, 21, etc.).

4.2.2.1. Stability of the degradation character

After the isolation procedure the isolates were cultivated and preserved in the first phase of the work on complex media providing sufficient cell densities for culture maintenance. As a result of this preservation and repeated cultivation of the isolates on complete media, 19 isolates irreversibly lost their PHB-depolymerizing ability.

Due to this instability of the PHB degrading strains, an optimization of the culturing and preservation techniques was necessary. Instead of using rich media the mineral salt vitamin medium was modified to meet the specific growth requirements of the strains. In earlier experiments it was noted that the addition of sterile **l**aboratory **s**ludge **s**upernatant (*LSS*) (see chapter 7.2.4.), enhanced biomass formation without suppression of the depolymerization potential, i.e. a general increase in clear zone diameter was observed (fig. 4.14).

This fact probably points to a cometabolism since *LSS* did not support growth in absence of an additional carbon source and is probably a source of required growth factors occurring in the natural habitats of the isolate.

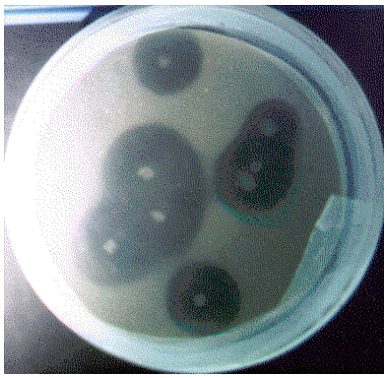


Fig. 4.14. Clear zone formation by strain 5a on PHB-MSV agar plates with 10 % *LSS* and 0.1 % yeast extract incubated for 5 days at 35 °C.

Applying the same screening and isolation protocol described under 4.2.1.3. and PHB-MSV-medium with a combination of different concentrations of *LSS* and the different co-substrates as well as yeast extract, seven stable isolates with a high degradation potential were obtained. The degradation potential of four stable isolated strains expressed as clear zone diameter on the different medium compositions is graphically illustrated in Fig. 4.15.

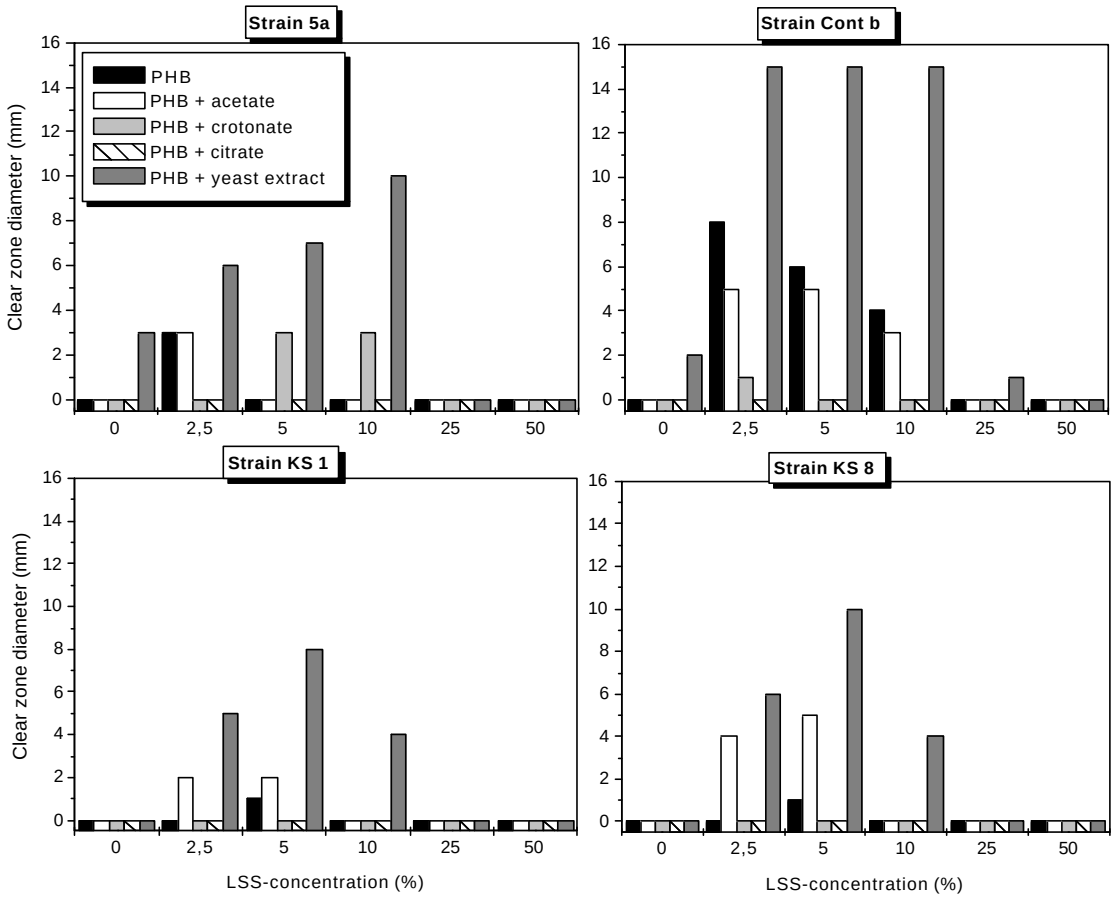


Fig. 4.15. Effect of different cosubstrates on PHB depolymerization by four stable isolates. Degradation potential is expressed as clear zone diameter (mm) after 10 days of incubation at 35 °C.

Working with unidentified isolates necessitates the use of diverse media compositions to meet the different nutritional requirements of the distinct individual strains. It must be stressed here, that the evaluation of degradation potentials is only reliable if different nutritional conditions are used. For example, if only MSV-PHB plates supplemented with citrate as a co-substrate were used, the isolates would be declared as being unable to depolymerize PHB as clearly demonstrated in Fig. 4.15. On the contrary, in the presence of crotonate or yeast extract the same organisms showed a good depolymerizing potential. Generally, a combination of yeast extract (0.1 %) and 2.5-10 % *LSS* is favorable. It must however be pointed out, that there exists no one optimal medium composition for all isolates since each organism has its own special growth requirements.

By adding the *LSS* to the MSV-medium the problem of low cell densities was solved. Thus complex media were not required for cultivation and/or preservation. In addition, the polyester depolymerizing character was maintained as the medium for preservation of the obtained culture collection also consisted of the MSV-medium supplemented with 0.1 % (w/v) yeast extract and 2.5 % (v/v) *LLS*.

4.2.2.2. Degradation studies using selected anaerobic bacterial strains

From the seven newly screened stable isolates two bacterial strains, namely strain 5a isolated from methane producing laboratory sludge and strain Cont b isolated from waste water sludge were selected as they comprised the highest PHB depolymerization potentials and were used for further investigations and identification.

Before using the two strains for mechanistic studies, an optimization of the medium composition was performed applying weight loss measurements as a more quantitative measure than clear zone formation (medium optimization with clear zones see above). In addition, the effect of rich medium components on PHB depolymerization was investigated.

Fig. 4.16 shows the experimental results of the quantitative determination of weight loss of PHB films in liquid culture with different medium compositions. Generally, the results can be correlated with clear zone investigations and show the same degradation tendency. Obviously, PHB degradation without supplements was not possible. On the other hand, as shown before yeast extract is definitely necessary for PHB degradation with these two

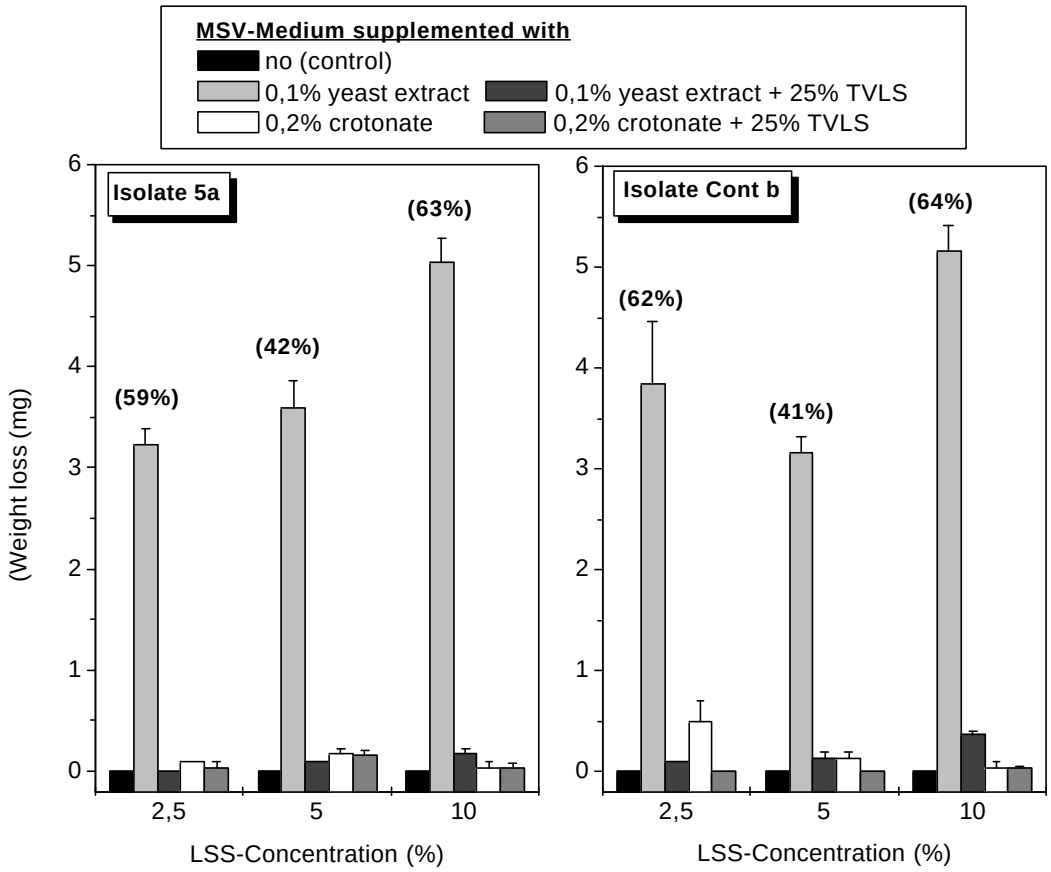


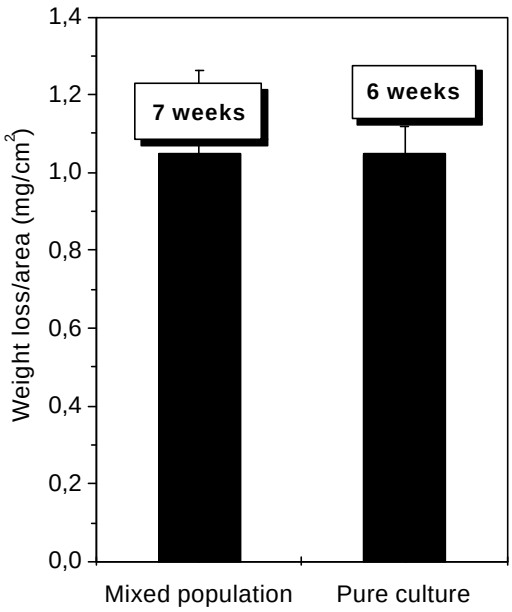
Fig. 4.16. Influence of medium supplementation with yeast extract or crotonate or alternatively the complementation with 25 % TVLS on biodegradability expressed as weight loss of polyester films. (m_i = 6 - 8 mg; ø = 9 mm; degradation surface area: 5.1 cm^2; n = 3 films per test) in liquid culture (Hungate tubes) with strain 5a and Cont b after 6 weeks at 35 °C.

strains since weight losses ranged from 41 – 64 % in presence of 0.1 % yeast extract. The addition of 25 % TVLS-complex medium in addition to yeast extract represses degradation significantly at all tested *LSS*-concentrations. This points to catabolite repression comparable to glucose. Crotonate supports polyester disintegration only to a limited extent regardless of the presence or absence of 25 % TVLS medium and weight losses lay only slightly above the control (0 – 6 %). The optimal medium composition and *LSS*-concentrations found for both strains were, therefore, 10 % *LSS* in presence of 0.1 % yeast extract. This medium was used for all other tests with the strains 5a and Cont b.

Keeping in mind, that polyester disintegration is a surface process, it was interesting that the weight loss data per surface area in the unidentified mixed populations (see chapter 4.1) are

directly comparable with the absolute weight losses under defined and optimized cultural conditions with the two selected strains as shown in Fig. 4.17.

Fig. 4.17. Comparison of the absolute weight losses per surface area of PHB films in unidentified mixed microbial populations (sludges and sediment) after a period of 7 weeks as well as two selected individual isolates (mean) after a six week long incubation in liquid MSV-medium supplemented with 10 % ***LLS*** and 0.1 % yeast extract at 35 °C.



In both cases weight losses are about 1.1 mg cm^{-2} after 7 weeks in the sludges and after 6 weeks with the purified strains. From this comparison it can be concluded, that the medium chosen represents well the nutritional conditions present in the anaerobic sludges and that degradation results obtained with the individual strains will have relevance to “natural” conditions.

4.2.2.3. Identification and characterization of two selected hydroxyalkanoate degrading isolates.

Two PHB-degrading strains, which exhibited the highest degradation rates were taxonomically identified.

16S rDNA partial sequence

The 16S rDNA analysis of the two selected strains 5a and Cont b revealed that both belonged to the phylogenetic group I of the genus *Clostridium* (*Clostridium* sensu stricto) after COLLINS ET AL. (1994). Both strains show 100 % sequence homology and are obviously strains of the same species with the highest sequence homology to *Clostridium botulinum* D (94.9 %), *C. homopropionicum* (94.1 %) and *C. scatalogenes* (94.1 %).

Strains that show a sequence similarity of less than 97 %, show a DNA-DNA reassociation value far below 70 % (STACKEBRANDT AND GOEBEL, 1994). In the phylogenetic definition of a

bacterial species it has been stated that a species would generally include strains with "approximately 70 % or greater DNA-DNA relatedness and with 5 °C or less ΔTm" (WAYNE ET AL., 1987). According to the analysis of 16S rDNA partial sequence (about 400 bases), DNA-base composition of 31.4 ± 0.1 mol %G+C; (n = 3) and the phenotypic properties, these two strains are representatives of a new species belonging to the genus *Clostridium*. In a cooperation with Dr. Hippe from the German Culture Collection in Braunschweig, Germany (DSMZ) the two isolates were further physiologically and biochemically characterized.

Morphological characters:

Cells of strain 5a are rods with slightly pointed ends, occur singly or in pairs and are occasionally bent (Fig. 4.18). Terminal, oval spores in swollen sporangia with a diameter of 1.35 µm were observed in medium DSM 503 with serine as a substrate (see 7.2.2. table 7.3).

Motility by peritrichous flagellation was detected in young cultures. Young cells of strain 5a grown in PY medium (see 7.2.2. table 7.3) stained Gram-negative; occasionally thicker cells were bluish.

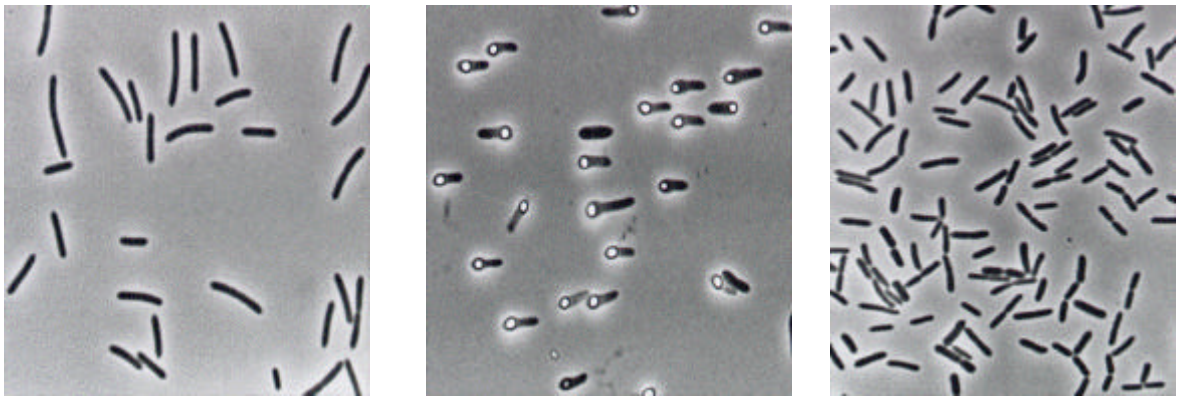


Fig. 4.18. Cell morphology of strain 5a and Cont b. a) Vegetative cells of strain 5a grown for 30 h on PY medium at 30 °C. **b)** Swollen vegetative cells of strain 5a containing sub-terminal/terminal endospores grown for 3 d on DSM-503 medium with serine incubated at 37 °C. **c)** Vegetative cells of strain Cont b grown for 8 h on PYG medium at 37 °C. Spores were not detected for this strain.

Colony characteristics and the morphological differences between strain 5a and Cont b are listed in Table 4.4.

Table 4.4. Morphological differences between strain 5a and Cont b.

Character	*Strain 5a*	*Strain Cont b*
Cell size:	0.54 - 0.9 x 2.25 - 6.3 µm	0.59 - 1.0µm x 2.25 - 5.0 µm
Spores :	+	not observed
Shape	oval	-
Position	sub-terminal; terminal	-
Sporangium	swollen	-
Colonies on:		
Columbia-sheep blood agar	2 mm, round, convex opaque, grayish white, shiny	1-2 mm, round, convex, little opaque, grayish white, shiny
PYG and TVLS (2 days)	1-2 mm gray, shiny, translucent round, entire margin	1 mm gray, shiny, translucent round, entire margin

Strain 5a is a strict anaerobe and no growth occurred in pink oxidized media; the presence of reductants in the medium is necessary. Maximal growth was detected at 37 °C and pH = 6.5. No growth occurred below 20 °C or pH = 5 and above 45 °C or pH = 8 (see appendix, fig. 9.1).

When yeast extract was omitted from the medium there was no growth in GMB or other media, i.e. yeast extract is required for growth. For all further work, at least 0.05 % yeast extract as a growth factor additive was routinely added to the medium.

Physiological and biochemical characterization:

Based on the phenotypic characters (see appendix, Table 9.1) the isolated strains 5a and Cont b are non-saccharolytic, non-proteolytic, non-gelatin hydrolyzing, and non-lipolytic microorganisms. Out of a wide range of carbon/energy sources tested, only those listed in Table 4.5 supported growth and both strains are obviously specialists. They grow best in a mineral-salt-vitamin medium with lactate. Even in PY medium lactate significantly stimulates growth.

Compounds utilized neither by strain 5a nor by strain Cont b are:

Na-malate, (1.0); Na_2-fumarate, (1.0); succinate, (1.25); alanine, (0.9); Na_2-glutamate, (1.0); Na-aspartate x H_2O, (0.17); ethylene glycol, (1.24); 1,2-propanediol, (1.0); 2,3-butanediol, (0.9); 1,4-butanediol, (0.9); ethanol, (2.0); propanol, (1.0); butanol, (1.0). Numbers in brackets represent the concentration (g l^{-1}) of the substrate.

Table 4.5. Growth of strain 5a and Cont b on various substrates in defined medium (DSMZ medium no. 503 with 0.1 % yeast extract added).

Substrate (g/l)	*Strain 5a (max. OD_{600nm})*	*Strain Cont b (max. OD_{600nm})*
Na-3-hyxdroxybutyrate (1.3)	0.222	0.196
Na-crotonate (1.0)	0.277	0.309
Na-lactate (2.5)	0.356	0.051
Na-pyruvate	0.062-0.113	0.056-0.130
Glycerol (1.0)		
+ Na-acetate (1.25)	-	0.025-0.067
Acetoin (1.0)	-	0.080
Serine (1.05)	0.098-0.122	0.44-0.134
Threonine (1.2)	0.277	0.40-0.092
Casaminoacids (5.0)	0.080-0.123	0.051-0.086

4.2.2.4. A novel group of obligate anaerobic bacteria belonging to the genus *Clostridium* degrading PHB:

Of the known 3-hydroxybutyrate and crotonate fermenting organisms, these two strains resemble *Clostridium homopropionicum* (DÖRNER AND SCHINK, 1990) and *Ilyobacter delafieldii* (JANSSEN AND HARTFOOT, 1990) in many respects. A comparison of some important characters is given in Table 4.6.

The different strains show similarities with respect to the fermentation products. The main products being acetate and butyrate, except for lactate as substrate which is fermented to acetate and propionate. In addition lactate induces 3-hydroxybutyrate fermentation for strain 5a, Cont b and *C. homopropionicum*. Obviously, dismutation of 3-hydroxybutyrate via crotonate to acetate and butyrate plays a central role in the metabolism of these strains.

C. homopropionicum was not known to utilize PHB; depolymerization of PHB by this strain was however proven in the present work. Concerning *I. delafieldii*, a PHB depolymerizing anaerobe, it must be mentioned that JANSSEN AND HARTFOOT (1990) never observed endospores for their isolate and hence placed it into the genus *Ilyobacter*. However, based on the recognized physiological similarities and the 16S rDNA sequencing performed by Dr. Hippe (DSMZ, Germany), this strain is clearly posed into the *Clostridium* cluster I and hence the re-classification of *I. delafieldii* as *Clostridium delafieldii* is proposed. It must be pointed out, that in fact all three species (strain 5a, *C. homopropionicum* and *C. delafieldii*) show a close taxonomical relationship.

Table 4.6. Comparison of some characteristics of strain 5a and Cont b, *Ilyobacter delafieldii* DSM 5704[t] and *Clostridium homopropionicum* DSM 5847[t]

Characteristics	*Strain 5a*	*Strain Cont b*	*Ilyobacter delafieldii*[1]	*Clostridium homopropionicum*[2]
Esculin hydrolysis	-	-	-	nd
Urea hydrolysis	nd	nd	-	nd
Gelatin hydrolysis	-	-	-	-
Lecithinase	-[3]	-[3]	nd	nd
Lipase	-[3]	-[3]	nd	nd
Sulfide from cysteine	nd	nd	-	nd
Indole production	-	-	-	nd
Oxidase	nd	nd	-	nd
Catalase	nd	nd	-	nd
2-hydroxybutyrate	nd	nd	-	
3-hydroxybutyrate	+	+	+	+
4-hydroxybutyrate	nd	nd	nd	+
4-Cl-butyrate	nd	nd	nd	+
Crotonate	+	+	+	+
Vinyl acetate	nd	nd	nd	+
Pyruvate	+	+	+	+
Fructose	-	-	-	+
Lactate	+	+	+	+
Acrylate	nd	nd	-	+
PHB	+	+	+	nd
Vitamins required	nd	nd	Pyridoxamine biotin	+
Yeast extract requirement	+	+	-	-
Growth on Columbia blood agar plates (BBL)	+	+	+	(+)
Reduction of sulphate	nd	nd	-	-
sulfur	nd	nd	-	-
thiosulphate	nd	nd	-	-
nitrate	-	-	-	-
pH range	5 - 8	5 - 8	5.9 - 8.6	5.6 - 8.3
optimum	6.5	6	7.8	7.2
Temperature range	20 - 45 °C	20 - 45 °C	10 - 41 °C	20 - 40 °C
optimum	37 °C	30 °C	40 °C	37 °C
NaCl range	0 - 50 g/l[5]	0 - 30 g/l[6]	0 - 40 g/l	0 - 10 g/l
Endospore observed	+	+	-	+
Gram staining reaction	-	-	-	-
Flagellation	peritr.	peritr.	peritr.	peritr.
Cytochromes	nd	nd	-	-
Fermentation products	acetate, (propionate)[4], butyrate	acetate, (propionate)[4], butyrate	acetate, (propionate)[4], butyrate	acetate, propionate[4], butyrate
$H_2 + CO_2$	-	-	H_2, CO_2	nd
G + C, mol%	31.4 %	31.4 %	29 %	32 %

[1] data from JANSSEN AND HARTFOOT, 1990; [2] data from DÖRNER AND SCHINK, 1990
[3] no growth on egg yolk agar plates according to HOLDEMANN ET AL. 1978
[4] lactate fermented to acetate and propionate
[5] not more than 10 %; [6] not less than 5 %
+, positive; (+), weakly positive; -, negative;
nd, not determined;

4.2.3. Individual strains degrading the aliphatic synthetic polyester PCL

From enrichment cultures with PCL incubated for 14 weeks at 35 °C in the different anaerobic sludges a total of 27 morphologically different anaerobic bacterial isolates were obtained on complex media.

A total of 16 isolates were capable of depolymerizing PCL. Co-substrate addition on PCL depolymerization was tested analogous to the isolates degrading PHB, but showed to have no significant impact with the exception of acetate. Only 6 strains formed clear zones on PCL-MSV mineral salt agar without supplementation with co-substrates, the remaining 10 isolates depolymerized PCL only in the presence of acetate. Table 4.7. lists the total number, the isolation source and the PCL-depolymerizing potential expressed as clear zone diameter of the screened isolates.

Table 4.7. Number and depolymerisation potential expressed as clear zone diameter of isolated PCL depolymerising anaerobes. The influence of acetate addition on PCL-break down, as well as the source of isolation is given.

Total number of isolates forming clear zones on the different media:		**PCL medium supplemented with:**	
		no supplement	**acetate**
10		+	-
6		+	+
Isolation source	Number of isolates		
	Total	and clear zone diameter (mm)	
Laboratory sludge (LS)	6	4 (4-5mm)[a]	2 (4-6mm)
Waste water sludge (WWS)	9	5 (3-8mm)	4 (2-8mm)
Anaerobic river sediment (AS)	1	1 (2-4mm)	0
Total:	16	10	6

[a]) Numbers in brackets: clear zone diameter (mm) formed on PCL supplemented mineral salt agar plates incubated for two weeks at 35 °C.

As expected from the observation of PCL-weight loss in the sludges (see chapter 4.1), the three different microbial sources are inhabited by PCL depolymerizing organisms. However, the technically relevant sludges (***LS*** and ***WWS***) harbor a broader spectrum of isolates (94 %) compared to the natural habitat (***AS***) harboring only one isolate.

The degradation potential of the 16 PCL depolymerizing isolates was directly quantitatively determined by monitoring weight loss of PCL-films in liquid MSV medium supplemented with and without 25 or 50 % TVLS-medium (fig. 4.19). As shown with PHB degradation (Fig 4.15, 4.16) the results obtained by weight loss of polyester films can be correlated with clear zone investigations and show the same tendency in degradation behavior.

The organisms responded differently to the medium composition with respect to their degradation potential and as expected there exists no general optimal degradation medium for all the different organisms. For 8 isolates highest (or at least equal) weight losses were recorded in mineral salt medium without any addition of complex medium.

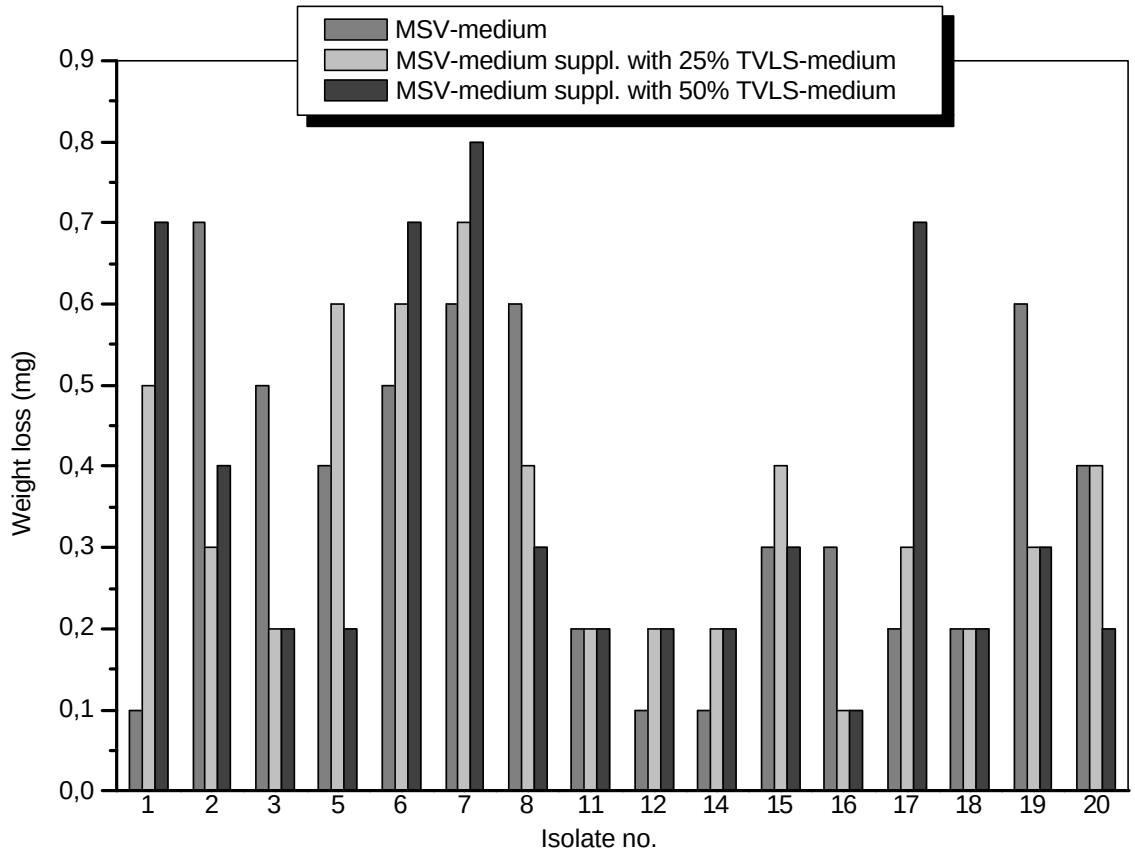


Fig. 4.19. Biotic hydrolysis of PCL films by 16 isolated anaerobic stains in MSV-media with different supplements after 3 weeks at 37 °C. (PCL-films; n = 3 parallel films per test; m_i: 18 - 28 mg; ∅ = 0,9 mm; surface area: 1.3 cm^2).

Strain PCL 6, a waste water sludge isolate, and PCL 7, isolated from methane producing laboratory sludge, however, exhibited the highest overall weight losses in the three different media and were, therefore, selected for further investigations.

4.2.3.1. Degradation studies using the two selected strains

For both strains a more detailed degradation medium optimization was performed (fig. 4.20). Generally, it was noticed that the higher the TVLS-complex medium content the higher were the measured weight losses.

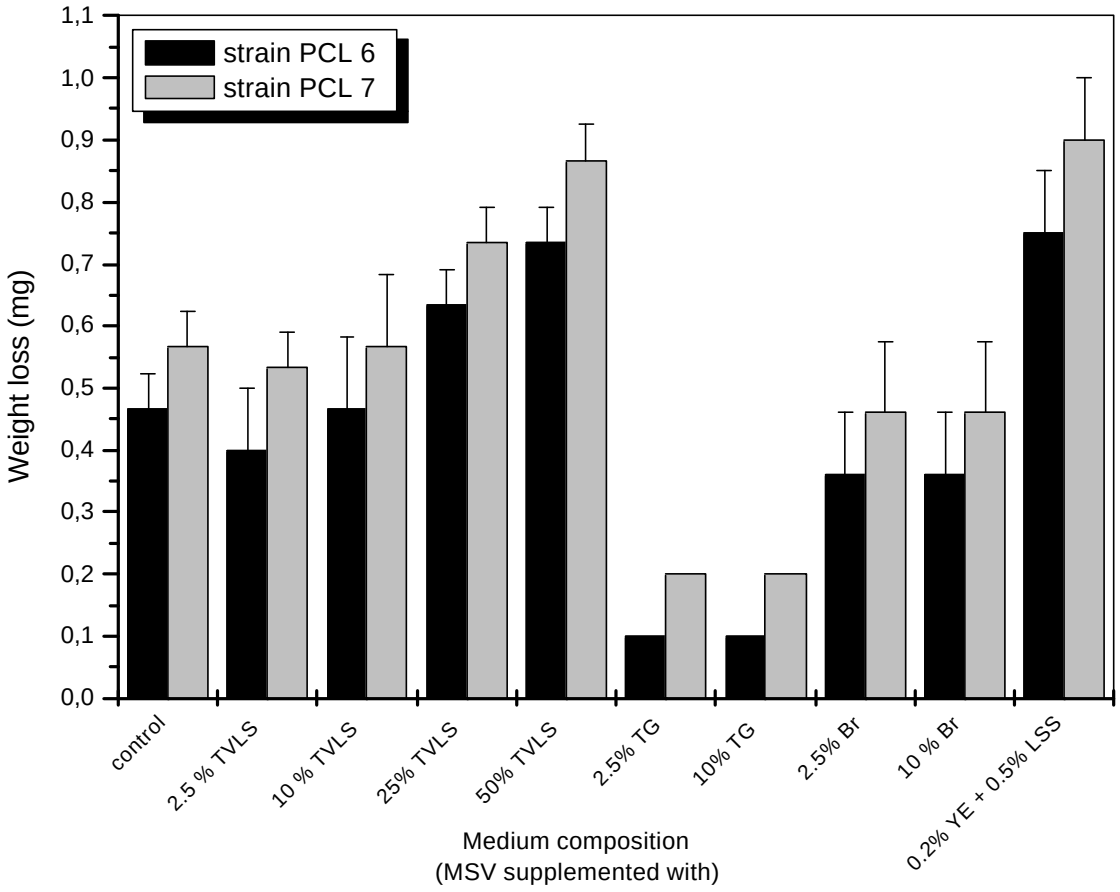


Fig. 4.20. Biotic hydrolysis of PCL films by 2 selected isolates PCL 6 and PCL 7 in MSV-media with different supplements after 3 weeks at 37 °C. (PCL-films; n = 3 parallel films per test; m_i: 18 – 28 mg; ∅ = 0,9 mm; surface area: 1.3 cm^2).

The addition of other complex media such as the TG or Br medium rendered weight losses lower than the control values. On the other hand, the addition of 0.2 % YE and 0.5 % *LSS* compensated the presence of complex media components and, therefore, this medium was used for further investigations. Obviously only components of the added yeast extract or growth factors of the *LSS* which are also present in the complex medium were responsible for the high degradation potential.

4.2.3.2. Identification and characterization of two selected PCL degrading isolates.

Two PCL-degrading strains, which exhibited the highest degradation rates were taxonomically identified.

Genetic identification

According to the 16S rDNA analysis the two selected strains PCL 6 and PCL 7, show only a minor similarity of 91.6 % to *C. acetobutylicum,* and an even lower relationships of 87.9 % to *C. collagenovorans* and only 86.8 % to *C. sardiniesis* . Both isolates are identical as shown by the 100 % sequence homology and represent two strains of a species belonging to the phylogenic group I of the genus *Clostridium* (*Clostridium* sensu stricto) after COLLINS ET AL. (1994).

According to the analysis of 16S rDNA partial sequence (about 400 bases), the DNA-base composition of 31.2 ± 0.1 mol %G+C; (n = 3) and the phenotypic properties which differ clearly from those of *C. acetobutylicum* (see appendix, Table 9.3), these two strains represent a new species belonging to the genus *Clostridium*. In a cooperation with Dr. Hippe from the German Culture Collection in Braunschweig, Germany (DSMZ) the two isolates were further physiologically and biochemically characterized.

Morphological description:

Fig. 4.21. Shows the rod shaped cells of the isolates which occur mainly in pairs, rarely in chains and have rounded ends. Oval subterminal endospores can be seen in swollen, cigar-shaped sporangia. Sporulating cells at an early state of sporulation are granulous.

Fig. 4.21. Vegetative cells cultivated at 37 °C of strain PCL 6: a) grown for 20 h on TVLS medium, **b)** containing sub-terminal/terminal endospores grown for 6 d on milk agar medium and **c)** showing peritrichous flagella grown on TVLS medium for 20 h.

The morphological differences between strain PCL 6 and PCL 7 are listed in Table 4.8. (See also appendix, table 9.2; and fig. 9.2)

Table 4.8. Morphological differences between strain PCL 6 and PCL 7.

Character	*Strain PCL 6*	*Strain PCL 7*
Cell size:	0.68-0.9 x 3.6-6.8 (9) µm	0.9µm x 3.0-10.4 µm
Filaments	31.5µm	-
Cell morphology:		
shape	rods, mainly pairwise, rarely chains (on TVLS medium)	rods, mainly pairwise, rarely chains (on PYG medium)
ends	rounded	rounded
Granulous:	-/?	in swollen, sporulating cells at early state of sporulation (milk agar)
Spores :	Not observed	+
shape	-	oval
position	-	sub-terminal
sporangium	-	Swollen, cigar shaped
Motility:	+	+
Flagellation:	peritrichous	peritrichous
Pigmentation:	colonies, culture, cell sediment: not pigmented	colonies, culture, cell sediment: not pigmented
Colonies on:		
PYG (2 days)	3-5 mm diameter strongly white, shiny, unbonate (irreg. surface) little irregular opaque	3-5 mm diameter strongly white, shiny, unbonate (irreg. surface) little irregular opaque

Physiological and biochemical characterization:

Both strains grow in mineral salt medium (without yeast extract and peptones) with 1 % glucose and vitamins (GMB). Yeast extract and peptones are not required for growth, but a complex vitamin mixture (Wolin vitamin mixture) is essential for growth in a mineral medium.

The physiological and biochemical characterization revealed that both strains are saccharolytic clostridia which utilize a large sugar spectrum, form butyric acid and weakly hydrolyze gelatin (see appendix; Table 9.2).

4.2.4. Isolation of SP 3/6 and SP 4/6 degrading anaerobes

First trials to isolate SP 3/6 or SP 4/6 hydrolyzing anaerobes from 14 weeks old enrichment cultures failed. Only after 18 month of enrichment, isolates depolymerizing aliphatic polyesters were obtained from SP 3/6 enrichment cultures.

From a total of 15 morphologically different anaerobes isolated from 18 month old enrichment cultures with SP 3/6, 9 strains were capable of SP 3/6 depolymerization. Comparable to PHB disintegration, the strains exhibited different co-substrate requirements for clear zone formation. Especially yeast extract enhanced polyester hydrolysis.

Table 4.9. lists the total numbers, isolation source and SP 3/6 depolymerizing potential (expressed as clear zone diameter) of the screened isolates. Interestingly, no isolates were obtained from the natural environment (anaerobic river sediment) enrichment cultures, and most isolates originated from ***WWS*** (6), exhibit a higher degradation potential and seemed to be more versatile concerning the co-substrates supporting clear zone formation.

Table 4.9. Screening of SP 3/6 degrading organisms from different enrichment cultures after 18 month of incubation at 35 °C.

Microbial source	**SP 3/6 as enrichment substrate No. of organisms screened on SP 3/6 medium supplemented with:**					
	Total	No suppl.	Acetate	Crotonate	Yeast extract	LLS
Laboratory sludge (LS)	**3**	1 (1-5 mm)	1 (15-20 mm)	0	1 (1-10 mm)	0
Waste water sludge (WWS)	**6**	6 (5-22 mm)	4 (1-9 mm)	2 (1-15 mm)	6 (1-10 mm)	2 (1-4 mm)
Anaerobic river sediment (AS)	**0**	0	0	0	0	0
Total no. of isolates	**9**	**7**	**5**	**2**	**7**	**2**

4.2.4.1. Degradation studies using a selected strain

One isolate from waste water sludge, namely strain KS-SP4/6, depolymerizing SP 3/6 as well as SP 4/6 (Fig. 4.22) was selected for further investigations with the aim of determining the substrate specificity and depolymerizing potential of this particular organism.

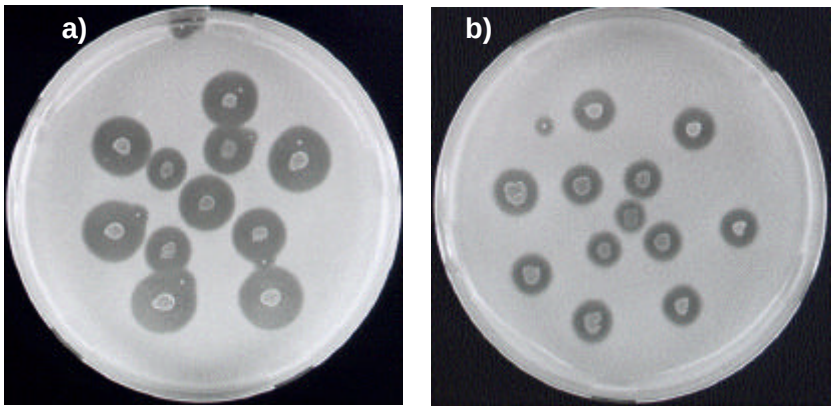


Fig. 4.22. Clear zone formation on a) SP 3/6- and b) SP 4/6-containing MSV-agar plates with strain KS-SP46 after 3 days at 37 °C.

The degradation potential expressed as clear zone diameter on different polyester mineral salt vitamin agar plates with strain KS-SP4/6 is given in Fig. 4.23. The isolate obviously has a broad polyester depolymerizing spectrum. From the six tested synthetic polyesters only the aliphatic-aromatic copolyester BTA 40:60 with a high aromatic content is not depolymerized. The natural hydroxyalkanoates, PHB and PHBV, also resist the enzymatic attack by this isolate.

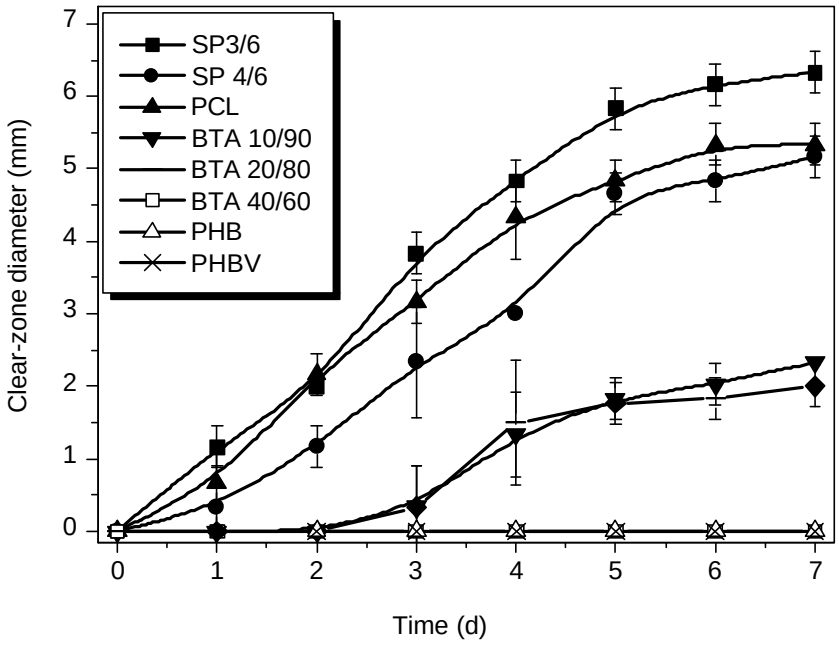


Fig. 4.23. Degradation potential of strain KS-SP4/6 measured as clear-zone diameter on different polyester containing MSV-agar plates over a period of 7 days at 37 °C.

Since the isolate has such a broad polyester-substrate spectrum the question arose if the strain metabolizes the depolymerization products and what the growth substrates of this

strain are. As shown in Fig. 4.24 the growth substrates of KS-SP4/6 are rather limited and are glycerine, fructose, glucose, erythritol and best of all mannitol.

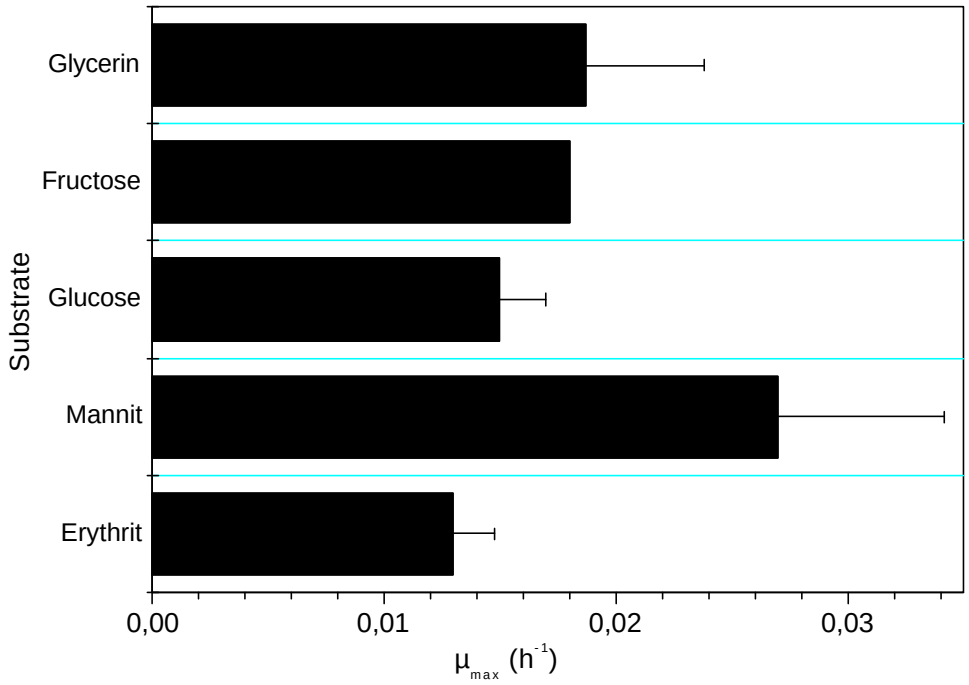


Fig. 4.24. Specific growth rates (mean of three parallel tests) of strain KS-SP4/6 on different growth substrates at an incubation temperature of 37 °C. Not used were: formate, pyruvat, valerate, 3-hydroxybutyrate, 2,3-butanediole, lactate, lactose, ethanol, methanol, CO_2/H_2O (80:20%). Concentration of the C-source: 5 g l^{-1} from a 10 % stock solution.

On the other hand, strain KS-SP46 did neither grow on 1,3-propanediol, 1,4-butanediole, adipate, terephthalate, nor caproate. This means that the strain is capable of depolymerizing synthetic polyesters but does not metabolize the depolymerization products, i.e. the monomers of the polyesters SP 3/6, SP 4/6 and PCL or BTA. The involved depolymerizing enzyme seems to be an unspecific degrading enzyme, probably an unspecific lipase. This unspecific enzyme is induced by the presence of the synthetic aliphatic polyesters which seem to act as gratuitous inducers.

4.2.4.2. Identification and characterization of the selected SP 3/6 degrading isolate.

The strain KS-SP 4/6 was taxonomically identified.

Genetic identification:

According to the 16S rDNA analysis the selected strain KS-SP 4/6 shows only a minor similarity to other known strains: *Sporomusa paucivorans* (91.0%), S*poromusa termitida*

(89.4%), *Sporomusa silvacetica* (90.8%) and even lower to *Dendrosporobacter quercicolus* (88.2%).

Morphological description:

Cells are vibrio shaped in growing cultures and form slightly curved long rods during the fermentation phase (fig. 4.25a). Spores are readily formed in swollen sporangia on rich media such as PYG and TG medium or on mineral salt media containing fructose (fig. 4.25b). Cells are motile and reveal a Gram-negative cell wall.

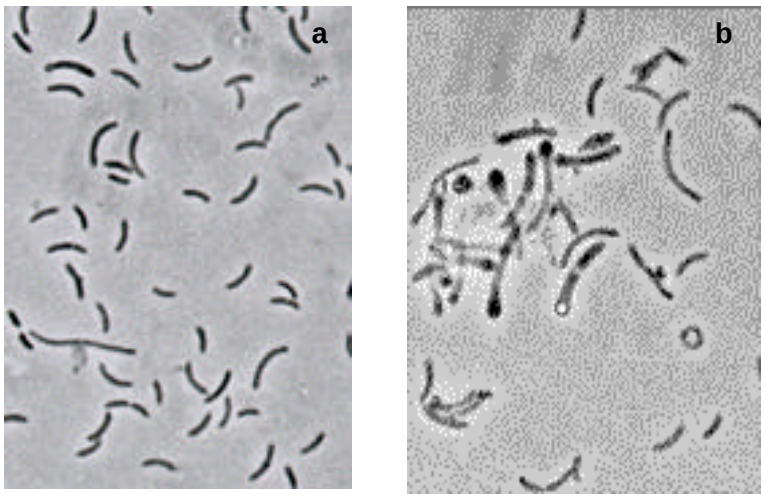


Fig. 4.25. Vegetative cells of strain KS-SP4/6: a) grown for 24 h and **b)** for 48 h showing terminal endospores on mineral salt medium containing fructose at 37 °C

Physiological and biochemical characterization:

Strain KS-SP4/6 proved to be a mesophilic organism with good growth between 20 - 50 °C and an optimum at 37 °C (see appendix, Fig. 9.3); The pH-range for growth lay between 5 – 8.5 with an optimum at pH = 6.8. The presence of yeast extract (at least 0.05 %) in the cultivating media was required.

A limited number of carbon and energy sources are utilized, mostly sugar alcohols. Synthetic polyesters are non-specifically depolymerized and the end products of depolymerization are not used as growth substrates. The strain follows in its fermentation pattern the acetate-propionate producing fermentative organisms as clearly shown in table 4.10.

Table 10. Fermentation balances of strain KS-SP4/6 grown on various carbon sources.

Products	Product recovery (mol/mol substrate) from					
	Glucose	Fructose	Mannitol	Xylitol	Erythritol	Glycerol
Propionic acid	1.19	1.04	1.19	0.90	0.98	0.79
Acetic acid	0.6	0.55	0.55	0.40	0.29	0.24
CO_2	0.75	0.67	0.55	0.40	0.35	0.37
H_2	0.02	0.05	0.21	0.15	0.16	0.23
Carbon	**0.92**	**0.82**	**0.87**	**0.78**	**0.96**	**1.08**
Avail. hydrogen	**0.90**	**0.80**	**0.83**	**0.75**	**0.91**	**0.97**

According to the analysis of 16S rDNA partial sequence (about 400 bases), and the phenotypic properties which differ clearly from those of the different *Sporomusa* spp., the strain was classified as a new species of the new genus *Propionispora* recently described by BIEBL ET AL. (2000).

4.2.5. Screening for individual strains degrading the synthetic aliphatic-aromatic copolyester BTA 40/60

From a total of 15 morphologically different bacterial strains obtained from enrichment cultures of BTA 40:60 in the different mesophilic sludges, no isolates were capable of clear zone formation on BTA 40:60 plates. However, two unidentified and non-purified mixed cultures formed tiny clear zones on BTA 40:60 plates. After purification, clear zone formation was not observed any longer.

In order to investigate the anaerobic biodegradability of different BTA-polymers all the polyester degrading isolates (chapter 4.2.) were tested for their capability to depolymerize BTA polyesters with lower mol% ratios of aromatic to aliphatic constituents (fig. 4.26). The obtained results suggest, that only aliphatic sequences within the statistical polyester with low aromatic content (mol%) of BTA are depolymerized by the different isolates, (i.e. BTA 10:90 and BTA 20:80). When the aliphatic sequences become short as in the case of BTA 32:68, BTA 40:60 and BTA 38:62, the polyester chain mobility is decreased and probably becomes inaccessible to the active center of enzymes like lipases (MARTEN, 2000). Consequently, the polyester becomes resistant to attack by single isolates, probably due to an inaccessibility to the enzyme system. It seems, that primarily the aliphatic sequences are cleaved while those with an aromatic content remain unchanged. Since BTA 40:60 is a

statistical co-polymer, this would mean, that SP 4/6-like regions are depolymerized leading to a minor weight loss, while those containing aromatic sequences are left unattacked. This in turn, could explain the low weight losses of BTA 40:60 (caused by the depolymerization of the aliphatic, SP 4/6 like sequences available on the surface of the BTA-polyester film) in the enrichment cultures (mixed microbial populations).

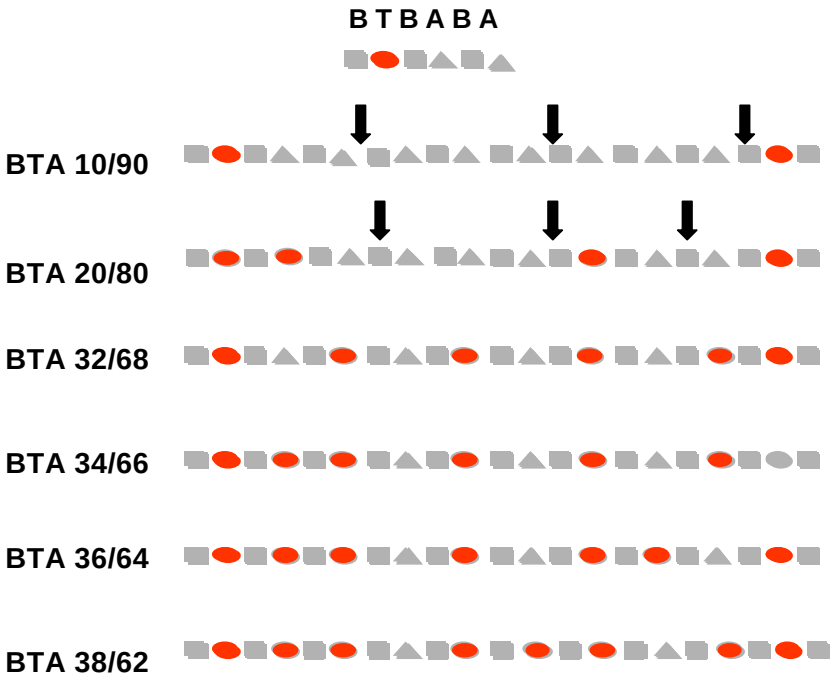


Fig. 4.26. Schematic presentation of the statistical aliphatic-aromatic copolyester BTA with different mol% ratios of aromatic to aliphatic constituents. Arrows represent hypothetical depolymerization sites.

Further attempts to isolate anaerobic strains capable of degrading BTA copolyesters with higher terephthalic acid contents were not performed due to time limitations.

4.2.6. Discussion

A total of 55 anaerobic bacterial strains were isolated from different enrichment cultures using different polyesters as an enrichment substrate. The question arose if these isolates can also degrade other polyesters than those from the corresponding enrichment culture and if the strains can be divided into separate groups depending on their substrate (polyester) specificities, i.e. which group of anaerobic organisms degrades which polyester.

Table 4.11 summarizes the total numbers of isolates capable of depolymerizing each one of the different polyesters. Interestingly, the total number of isolates capable of depolymerizing the different polyesters or polyester groups coincided with the degradation rates obtained

with the mixed microbial populations in the sludges. As the general succession of the depolymerization rate as well as number of depolymerizing organisms is as follows:

PHB > PHBV > PCL >> SP 3/6 ≈ SP 4/6 > BTA 10:90 ≈ BTA 20:80 (>> BTA 40:60).

Table 4.11. Substrate spectrum and number of depolymerizing isolates evaluated by clear zone formation on mineral salt agar plates supplemented with different polyesters.

Isolates from enrichments with	Number of tested isolates	Number of strains capable of degrading:							
		SP 3/6	SP 4/6	PCL	BTA 10:90	BTA 20:90	BTA 40:60	PHB	PHBV
SP 3/6	9	9	7	4	2	2	0	0	0
PCL	16	0	0	16	0	0	0	0	0
PHB	30	0	0	0	0	0	0	30	27
Total	**55**	**9**	**7**	**20**	**2**	**2**	**0**	**30**	**27**

From the results of table 4.11, it can be concluded that, the polyester degrading isolates (total number of 55) can be divided into three main groups: The PHB and PHBV degrading isolates are specialized to depolymerize only the natural hydroxyalkanoates and cannot attack synthetic polyesters and vice versa. The isolated and characterized natural PHA degrading anaerobic strains can be regarded as a group of closely related microorganisms of clostridia. The new strains 5a and Cont b representing strains of *Clostridium sp. nov*., isolated and characterized in this work showed several similarities to *C. homopropionicum.* Thus, this strain was checked for PHB degradation and it was proven during the present study that this organism can depolymerize PHB, too. Finally, *Ilyobacter delafieldii* which was already described as a PHB degrader in the literature, obviously must be re-classified as *Clostridium delafieldii* according to the new taxonomic data.

Strain 5a and Cont b are metabolically highly restricted but metabolize the depolymerization product (monomer) 3-hydroxybutyrate. The organisms seem to be specialized on PHB degradation and metabolization. The involved enzyme is most probably a specific PHB depolymerase, an enzyme which has been intensively investigated for aerobic microorganisms. The enzyme seems to be induced by PHB and catabolically repressed by the presence of glucose (see chapter 3.6.3). A more detailed investigation of the PHB depolymerizing enzyme regulation is given in chapter 4.4.2.

PCL degrading strains are also specialists since they only showed depolymerization activity towards PCL. No organism originally screened on PCL, degrades SP 3/6, SP 4/6 or BTA. Since the characterized strains *Clostridium* sp. nov (strains PCL 6 and PCL 7) ferment a wide sugar spectrum proved however to be lipase negative and did not metabolize the depolymerization products, i.e. the monomers, the involved enzyme system must be a hydrolyzing enzyme, which unspecifically also depolymerizes PCL probably due structural similarities between its depolymerization products and those of another structurally similar polymer, such as for example cutin. MURPHY ET AL. (1996) showed that PCL dimers and trimers are structurally similar to natural inducers of cutinase. Additionally, literature on aerobic PCL-degradation gives evidence about the involvement of cutinases in PCL depolymerization (Nishida and Tokiwa, 1994b; MURPHY ET AL. 1996). This would mean that the products of PCL depolymerization succeed - due to structural similarities to cutin depolymerization products - in binding to the repressor protein (which normally binds to the depolymerization products of cutin) and thus induces the transcription of the cutinase (see chapter 3.6.3).

In contrast, strains isolated from SP 3/6–enrichment cultures (total of 9) show a wide substrate spectrum within the synthetic polyesters but do not hydrolyze the hydroxyalkanoates. The selected characterized *Propionispora* sp. nov. (strain KS-SP4/6) also does not metabolize the depolymerization products of the polyesters SP 3/6, SP 4/6, PCL or BTA. The involved depolymerizing enzyme seems – comparable to the PCL degrading enzyme - to be an unspecific degrading enzyme, probably an unspecific lipase induced by the presence of gratuitous inducers, i.e. the synthetic aliphatic polyesters. KLEEBERG (1999) documented a similar situation were aerobic BTA depolymerizing strains of *Thermomonospora fusca* secreted an extracellular hydrolase which unspecifically depolymerized the copolyester and several other synthetic aliphatic polyesters. The resulting depolymerization products were not metabolized by the strains.

Obviously, at least three different enzyme systems are involved in the anaerobic degradation of the different polyesters. This observation is congruent to the aerobic situation where also three different kinds of hydrolases are discussed to be involved in polyester degradation.

In addition it is worth noting, that the natural polyesters and the moderately biodegradable PCL did not require extended enrichment periods for the isolation of degrading strains. Yet, for the isolation of isolates depolymerizing the synthetic polyesters SP 3/6, SP 4/6 and BTA

10/90 as well as BTA 20/80 an enrichment period of 18 was necessary. In the present case, probably a nonspecific lipase producing organism had to adapt its enzyme regulation mechanisms to the synthetic and unusual polyester substrate.

Most interestingly is the finding, that all the organisms selected for identification are not yet described species. Although the genus *Clostridium* has grown to be one of the largest genera among prokaryotes (CATO AND STACKEBRANDT, 1989), obviously many of them are still undiscovered. The isolated organisms are highly specialized (e.g. strain 5a) or degrade substrates such as synthetic polyesters which are not considered as conventional microbial substrates (e.g. strain PCL 6; strain KS-SP4/6) and are hence only isolated under specific and selective (nutritional) growth conditions.

It is important to mention, that carbon catabolite repression (CCR) by glucose was observed for the organisms degrading hydroxyalkanoates, i.e. that cells sense the presence of a favorable carbon source, in this case glucose, and transmit the information to the relevant control units. Consequently, PHB as a C-source is not depolymerized to act as a catabolite. In clostridia, (four out of five identified strains are clostridia) there is no evidence for the involvement of cAMP in CCR, characteristic for Gram negative bacteria, as is the case in *E. coli* (see 3.6.3.). However, some recent evidence indicates that clostridia do share some features with other Gram positive bacteria. The most general mechanism seems to be the "inducer expulsion" (SAIER ET AL., 1996; MITCHELL, 1998; STÜLKE AND HILLEN, 1999) which is believed to occur in *Clostridium* sp. P262 (DIEZ-GONZALEZ AND RUSSELLL, 1996), *C. acetobutylicum* DSM 792 (BEHRENS ET AL., 1997), and *C. beijerinckii* NCIMB 8052 and *C. pasteurianum* (MITCHELL, 1998). This mechanism is mediated by the HPr-protein (the phospho-carrier protein of the phospho-enol-pyruvate (PEP) : sugar phosphotransferase system (PTS)), or more accurately the kinase which phosphorylates it. This protein is responsible for the regulation of PTS-activity and of the non-PTS-transport systems, activation of sugar-phosphate phosphatases, and control of transcription of catabolic operons (see fig. 3.6).

Inducer expulsion represents a mechanism by which inducing compounds are readily expelled from the cytoplasm in the presence of a readily metabolizable sugar substrate. Upon the addition of glucose and subsequent formation of the HPr-phosphate complex at the serine-46 position (HPr-Ser46-P), the sugar-phosphate is readily dephosphorylated by a sugar-phosphate phosphatase and the free sugar is exported. This process is dependent on

ATP and a glycolytic intermediate and is elicited by PTS sugars other than glucose (Fig. 4.27).

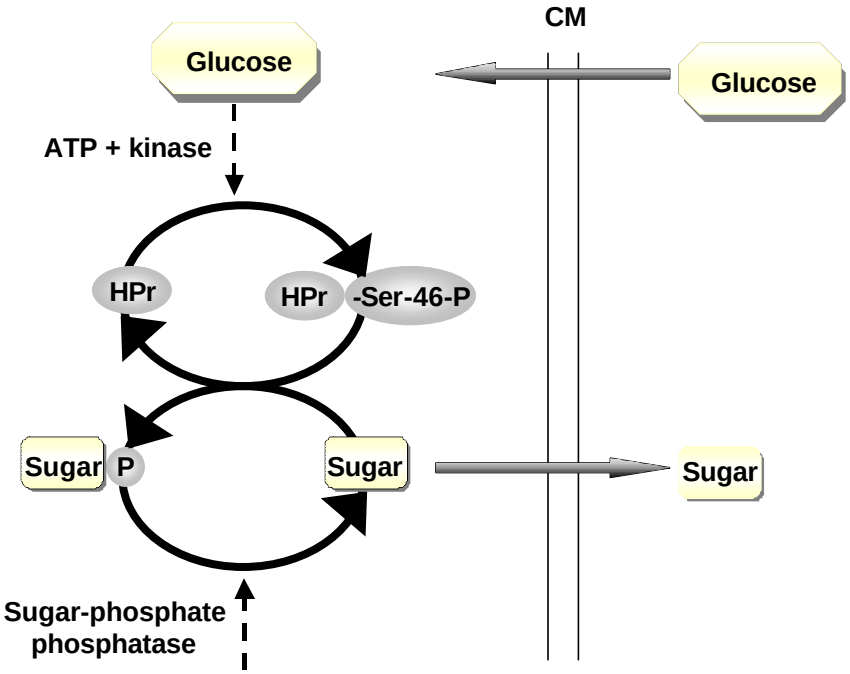


Fig. 4.27. Schematic representation of the inducer expulsion mechanism occuring in *Lactobacillus lactis*, *Streptococcus pyogens*, *S. bovis*, *Enterococcus faecalis* and *Clostridium* sp.

A similar mechanism may be involved in the carbon catabolite repression by glucose observed by the isolated anaerobic organisms in the present study. Considering that the organism under test secretes a low basal and constitutive amount of the PHB depolymerizing enzyme, traces of the monomer 3-hydroxybutyrate are to be expected in the surrounding of the degrading strain. The monomer would consequently be transported into the cell and phosphorylated. However, due to the concomitant presence and uptake of glucose into the cell, the HPr-protein would be phosphorylated and 3-hydroxybutyrate simultaneously dephosphorylated. The free 3-hydroxybutyrate would consequently be expelled out of the cell again. Thus, despite the presence of traces 3-hydroxybutyrate which could act as an inducing catabolite, the genes encoding the depolymerizing enzyme would not be transcribed to an significant amount. It must however be stated, that additional data and precise investigations are necessary to characterize the involved system. The characterization of the regulatory function and the repressive mechanisms occurring in these organisms is definitely challenging.

Catabolite repression by glucose observed for the organisms degrading hydroxyalkanoates resulted in problems with preservation of the organisms and/or instability of the degradation character. The extrachromosomal codation of such a character may be a possible reason for

these observations. WIEGAND ET AL. (1999) studying the aerobic degradation mechanism of BAK 1095 (a biodegradable random copolymer of polyester amide developed by Bayer AG) also observed the loss of the degradation ability by the isolated degraders when there was temporarily no selection pressure and the organisms were not forced to grow with BAK 1095 as the sole source of carbon. On the other hand, catabolite repression was not observed for the organisms depolymerizing synthetic polyester and rich medium addition enhanced polyester depolymerization, thus no problems with preservation of organisms and/or instability of degradation character occurred.

Generalizing it can be stated, that the degradation process is dependent on the efficiency and frequency of specialized organisms. Different sludges may contain similar anaerobic bacterial species, e.g. strains belonging to the same species such as strain 5a isolated from laboratory sludge and Cont b (waste water sludge) which depolymerize - in this case - PHB and differ only slightly in their characters. Yet, the sludge compositions vary and consequently the degradation efficiency obtained with similar organisms but different nutritional environments may alter. In addition, the degradation potential of the different isolated organisms was greatly influenced through variation/optimization of the growth medium, since the different organisms exhibit different nutritional requirements. Comparably, the different sludges and sediment used in the comparative degradation studies vary in their composition and may or may not satisfy the different nutritional requirements of the degrading organisms, thereby limiting their degradation capabilities. As has been demonstrated by the present data, the presence of a readily available C-source such as glucose may catobolically repress polyester degradation or lead to the loss of the degrading character as in case of PHB depolymerization (glucose or similar substrates are constituents of sludges). This may lead to heterogeneous degradation results for the same polyester in different sludges even though they inhabit the same or at least similar organisms. Therefore, results obtained with unidentified mixed microbial populations can only be interpreted if the factors influencing the responsible degrading organisms are known and understood.

4.3. Evaluation of the anaerobic biodegradability of PHB with the selected anaerobic microorganism *Clostridium* sp. nov. (strain 5a)

With the aim of investigating the degradation mechanism as well as gaining more information about the enzyme system and the parameters influencing the degradation process, it was intended to develop an advanced PHB degradation test system. As a tool for systematic investigations under controlled and optimized cultural conditions, a characterized isolate was used, intending to reduce degradation times and to increase the accuracy of the data. For this purpose the bacterial strain *Clostridium* sp. nov. (strains 5a) isolated from methane producing laboratory sludge was chosen, because it specifically depolymerizes the polyester PHB and metabolizes the degradation product of PHB namely 3-hydroxybutyrate. In addition this strain has been well characterized in the previous chapter.

4.3.1. Comparison of PHB and PHB film degradation on agar plates

First, the time dependent degradation of PHB as well as PHBV films on agar plates was quantitatively investigated. In Fig. 4.28 the weight losses of the polyester films on mineral salt agar plates supplemented with 0.1 % yeast extract over a period of 11 weeks incubated anerobically at 35 °C in a glove box are shown.

An almost five fold higher degradation rate for PHB than for PHBV was obtained. These results correlate with those obtained in the sludges (fig 4.1 – 4.4, 4.9 & 4.10). Yet, these data are again in contradiction to aerobic PHB degradation results published in literature were PHB exhibits a lower biodegradability than PHBV due to its higher crystallinity (NISHIDA AND TOKIWA, 1993). The negative lag phases observed are considered as an artifact which may result from residues of agar medium or biomass/biofilm which have not been successfully removed during the film washing procedure.

It must be noted that the biological degradation rates under these experimental conditions were still too low (test time over 10 weeks) for further systematic investigations within a reasonable time scale. Obviously, this system poses some limitations indicated by the cessation of degradation after 30 % weight loss has been reached for PHB. Several factors might be responsible for this incomplete degradation being the buffer capacity of the test system, the depletion of important nutrients, surface properties and/or alteration, or the enrichment/inhibition of accumulating crystalline regions.

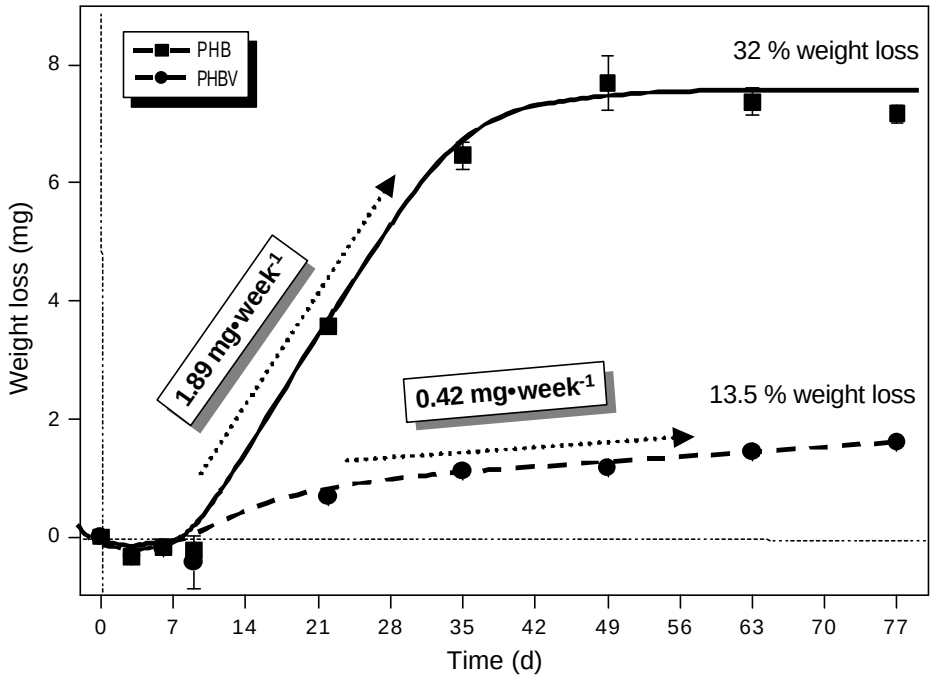


Fig. 4.28. Comparison of PHB- and PHBV-film degradation as a function of weight loss of polyester films. (m_i = 26 – 40 mg; ø = 25 mm; degradation surface area: 19,6 cm^2; n = 3 films per plate) on MSV-agar plates with *C.* sp nov. strain 5a at 35 °C. Numbers in boxes indicate the maximum degradation rates.

4.3.2. Scanning electron microscopy (SEM) analysis of PHB and PHBV film degradation by strain 5a

The ability of strain 5a to degrade PHB and PHBV films depends on the secretion of a specific enzyme that hydrolyses the polymer chains on the surface to water soluble products. Thus, morphological alterations of the polymer surface as a result of bacterial hydrolysis were examined by scanning electron microscopy. In addition, it was attempted to explain the cessation of degradation by surface properties or alteration.

PHB polyester films which had been partially degraded in the experiment described above were processed for scanning electron microscopy (see chapter 7.8.2.). The surface of the uninoculated control film incubated on sterile plates was smooth (fig. 4.29 a). This indicates that incubation of the PHB film on sterile media had no effect on the surface of the polymer. On the other hand, the film surfaces exposed to strain 5a for 7 weeks or 11 weeks had a rough appearance clearly different from that of the control (fig. 4.29 b - h). The surface of the films possessed numerous pits (arrow p) of varying sizes (fig. 4.29 b). However, zones of native film surfaces (arrow n) also appeared spread over the film (fig. 4.29 c - g). Interesting is the sheet wise or layer wise degradation behavior clearly indicated by fig. 4.29 e. The

upper layer which is less degraded (arrow l) comprises zones of native PHB while the subsequent deeper layer does not show such native material regions/islands.

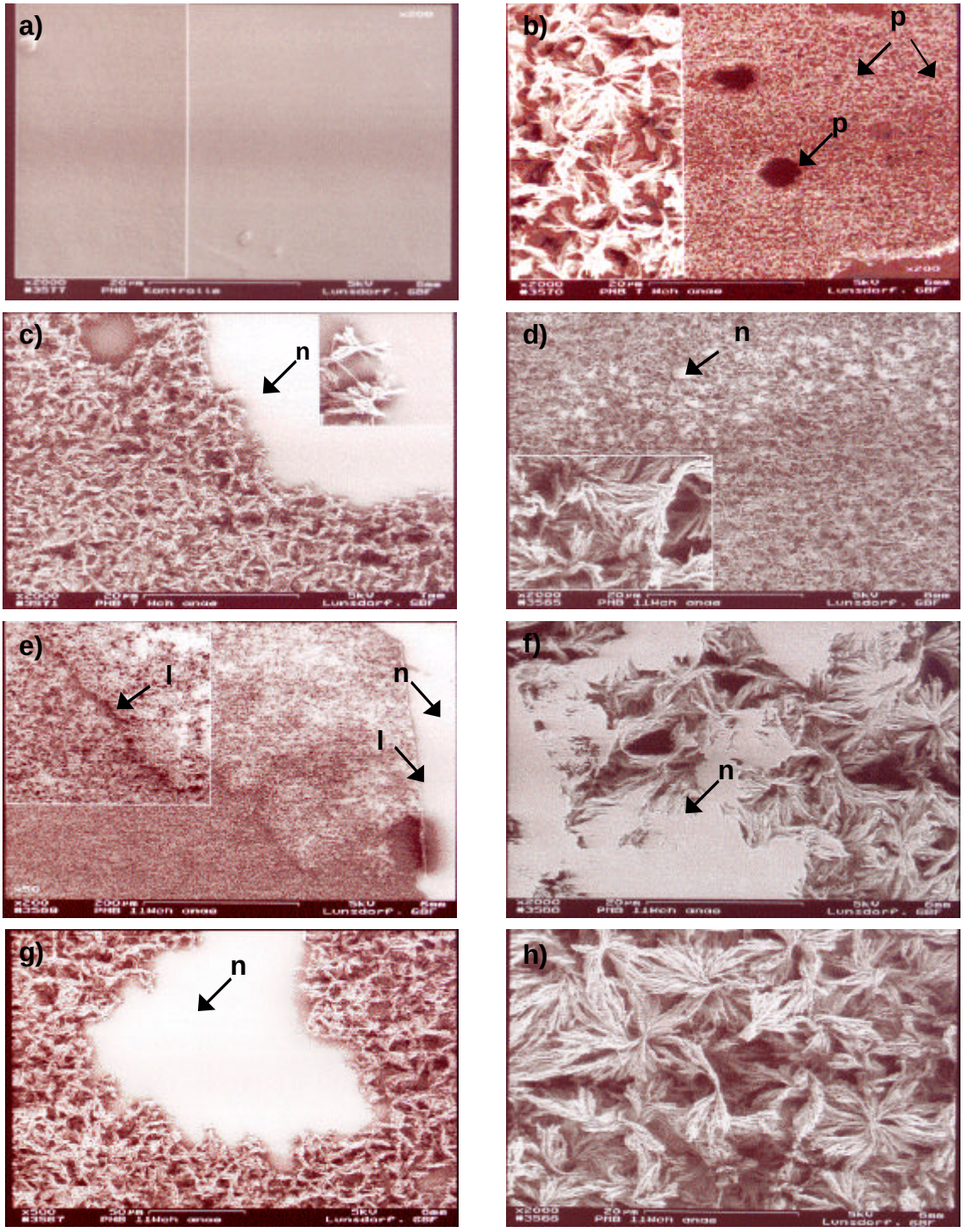


Fig. 4.29. SEM micrograph of PHB films exposed to strain 5a on mineral salt agar plates containing 0.1 % yeast extract at 35 °C. a) Surface of native sterile incubated control; b) and c) PHB film exposed for 7 weeks to strain 5a (film lost 7.3 mg (41 %): showing deep erosion pits (b) and spherulites. d) - g) PHB film after 11 weeks of incubation (film lost 7.2 mg) showing undegraded native materials as islands within the spherulites (d - h). p = pit; n = undegraded area; l = layer.

As with PHB, the surface of the uninoculated PHBV control film incubated on sterile plates was smooth and unaffected by sterile incubation (fig. 4.30 a). On the other hand, the film surfaces exposed to strain 5a for 7 weeks or 11 weeks had a rough appearance clearly different from that of the control (fig. 4.30 b - f). The surface of the films possessed no pits as those described for PHB (fig. 4.29 b). However, zones of native film surfaces (arrow n) also appeared spread over the film (fig. 4.30 b). Like PHB, a sheet wise or layer wise degradation behavior was observed here (fig. 4.30 f) comprising an upper layer which is less degraded

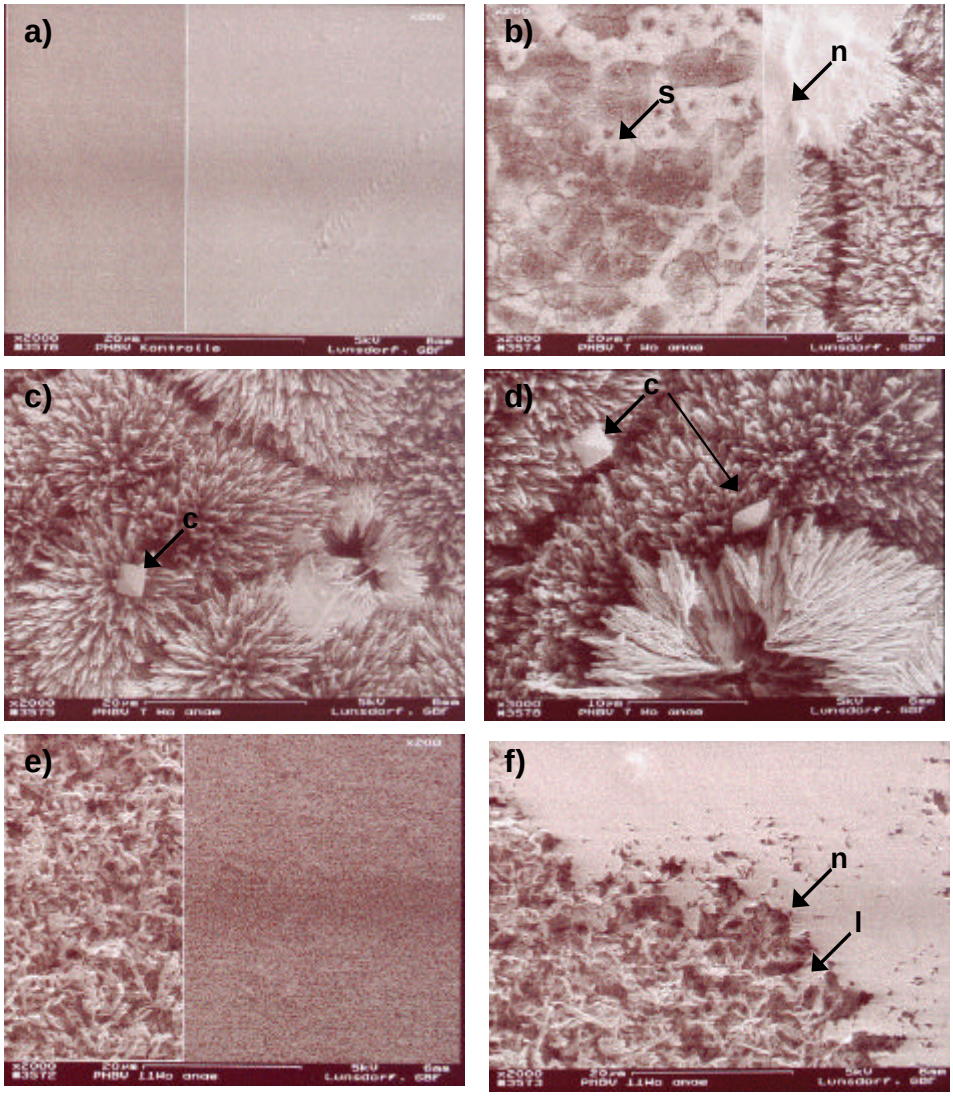


Fig. 4.30. SEM micrograph of: PHBV films exposed to strain 5a on mineral salt agar plates containing 0.1 % yeast extract at 35 °C: a) Surface of native sterile incubated control film; b -d) PHBV film exposed for 7 weeks to strain 5a, film lost 4.5 mg (30 %) of its initial weight; e) and f) PHB film after 11 weeks incubation, film lost 4,4 mg. s = spherulite; n = undegraded area; l = layer; c = crystal.

(arrow n) with zones of native PHBV, while the subsequent deeper layer (arrow l) does not show such native material. The crystals adherent to the polyester surface (fig. 4.30 c and d) may be the reason for the negative weight loss results pointed to under 4.3.1, fig. 4.28.

The ability of strain 5a to degrade PHB and PHBV films depends on the enzymatic hydrolysis of the polymer to water soluble products and polymer erosion proceeds via surface dissolution. It is obvious that amorphous regions of the polymer are more rapidly degraded than the crystalline ones. Similar results were found by KUMAGAI ET AL. (1992); NISHIDA AND TOKIWA (1993) as well as MOLITORIS ET AL. (1996). However, no logic explanation for the cessation of degradation of the polyester films (fig. 4.28) was deducible from the SEM micrographic examinations. Especially, since enough native and amorphous material remained on the polyester surfaces which could be further degraded.

4.3.3. Degradation experiment with PHB powder in a pH-controlled bioreactor

The incomplete degradation of PHB and the cessation of growth observed in the previous experiment prompted us to study the biodegradation efficiency of PHB in an improved and controlled homogenous test system, which additionally allows continuous sampling during the degradation process. In order to accelerate the degradation process, being a surface phenomenon, PHB powder was used instead of films to increase the available degradation surface area.

Fig. 4.31 shows the time course of five PHB degradation experiments in a pH-controlled reactor under comparable conditions and the average degradation curve of all tests. PHB degradation was measured by the decrease in PHB concentration determined spectrophotometrically (see chapter 7.11.) by the method of SENIOR ET AL. (1972).

In fact, in this system 1 g l^{-1} PHB powder was almost completely degraded within three days. This comprises a maximum degradation rate in terms of polymer mass loss of about 2680 mg $week^{-1}$ compared to about 2 mg $week^{-1}$ obtained on agar plates with films (fig. 4.28). This dramatic increase in degradation rate in the bioreactor makes this system a powerful tool for further investigations of the degradation mechanism. However, which parameter – the increase in polymer surface area or the pH-control – caused this high degradation rates could not be definitely decided from these data.

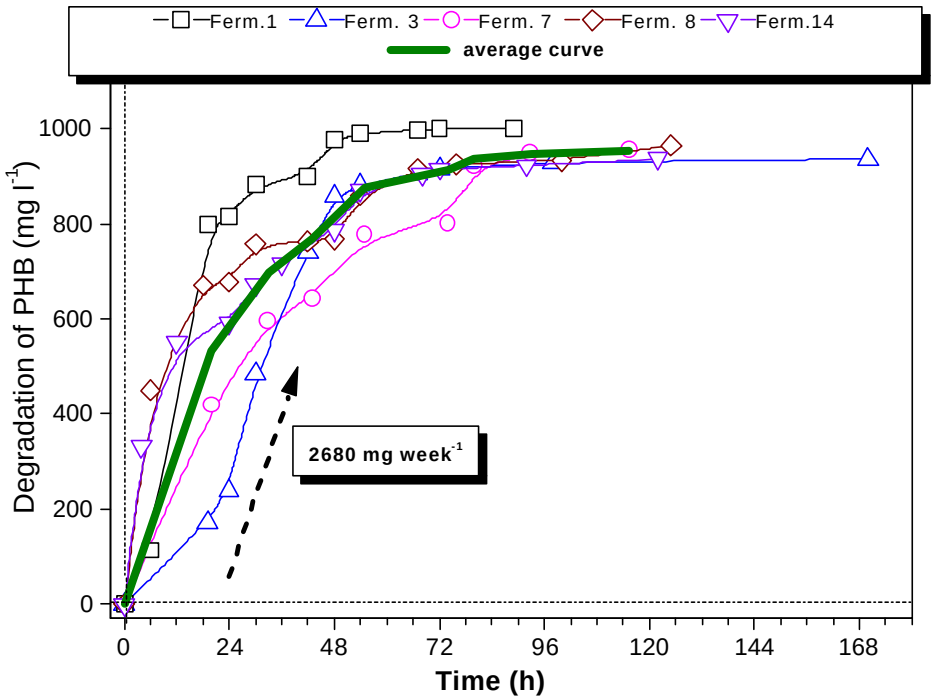


Fig. 4.31. PHB degradation elucidated as decrease in PHB concentration by strain 5a in a pH-controlled bioreactor. (MSV-medium; PHB (1 g l^{-1}) and 0.1 % yeast extract; pH = 6.8; 37 °C; stirred at 150 rpm; continuous N_2-gassing; working volume: 0.6 l). Number in box represents the maximum degradation rate.

Fig. 4.32 compares the absolute calculated degradation rates of PHB obtained with different test systems. Indeed, the degradation of PHB-powder in a pH-controlled bioreactor offers a reduction of the time scale for a degradation experiment from several weeks in sludges or on plates to a few hours in the controlled bioreactor.

Such a fast test system allows now the systematic and mechanistic investigations within a reasonable space of time and opens new perspectives/alternatives for investigations. Additionally, the data are more reliable since - compared to tests with sludges - the degradation medium is more or less defined and the amount of additional carbon source is calculable and remains constant. Furthermore, it must be pointed out that using polyester powder in sludges would make the recovery of the rest polymer very tedious and inaccurate. Even when films are applied the complete film recovery is not guaranteed if the polyester material already started to disintegrate. Performing degradation tests on plates solves this problem, since powder is principally applicable. Yet, the low buffer capacity and one point measurements limit the obtainable information. The use of a controlled bioreactor, as has been demonstrated, overcomes all these limitations.

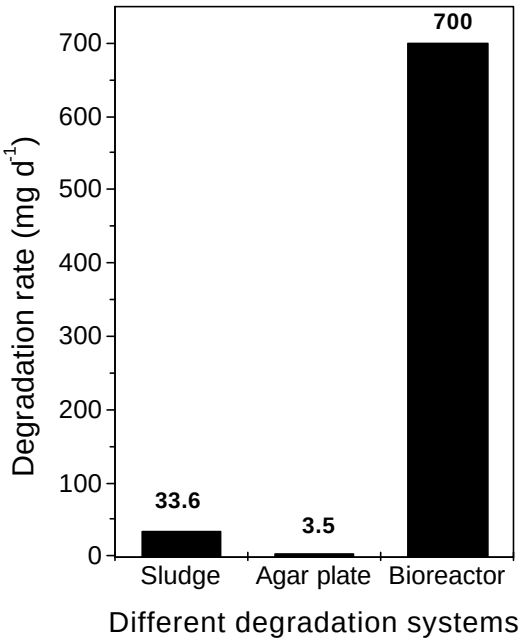


Fig. 4.32. Comparison of absolute degradation rates of PHB obtained with different test systems: the degradation of PHB films in sludges containing consortia of unidentified organisms, the film degradation with an identified and characterized PHB degrading isolate - strain 5a - on agar plates, and finally the degradation rate of PHB powder obtained with the same strain in a pH-controlled bioreactor.

4.3.4. Metabolic characterization of the PHB degradation process with strain 5a.

With this improved test system some basic questions about the mechanism of the PHB degradation with the particular strain 5a were to be investigated: What are the water soluble products of the depolymerization of the PHB chains by the extracellular enzyme? Are these intermediates detectable in the medium (intracellular fermentation of the intermediates could be so fast, that the intermediate concentration in the medium would be very low)? Can the organism metabolize the intermediates for energy production and what are the end products?

For this purpose cell growth determined as protein concentration and 3-hydroxybutyrate as well as end product formation was detected during PHB degradation in a bioreactor under controlled pH conditions (pH = 6.8). As clearly demonstrated in Fig. 4.33, 3-hydroxybutyrate was detected in the culture broth.

Obviously, growth of strain 5a proceeds concomitant with the appearance of 3-hydroxybutyrate which is the monomeric depolymerization product of PHB. Unfortunately, it still remains unclear, whether or not dimers and trimers are also produced as depolymerization products. However, it is obvious that strain 5a depolymerizes PHB into the monomer 3-hydroxybutyrate. In addition, acetate and butyrate were detected as the sole fermentation end products. This clearly indicates that PHB is definitely depolymerized to 3-

hydroxybutyrate which is subsequently intracellularly fermented to acetate and butyrate for energy production and cell growth.

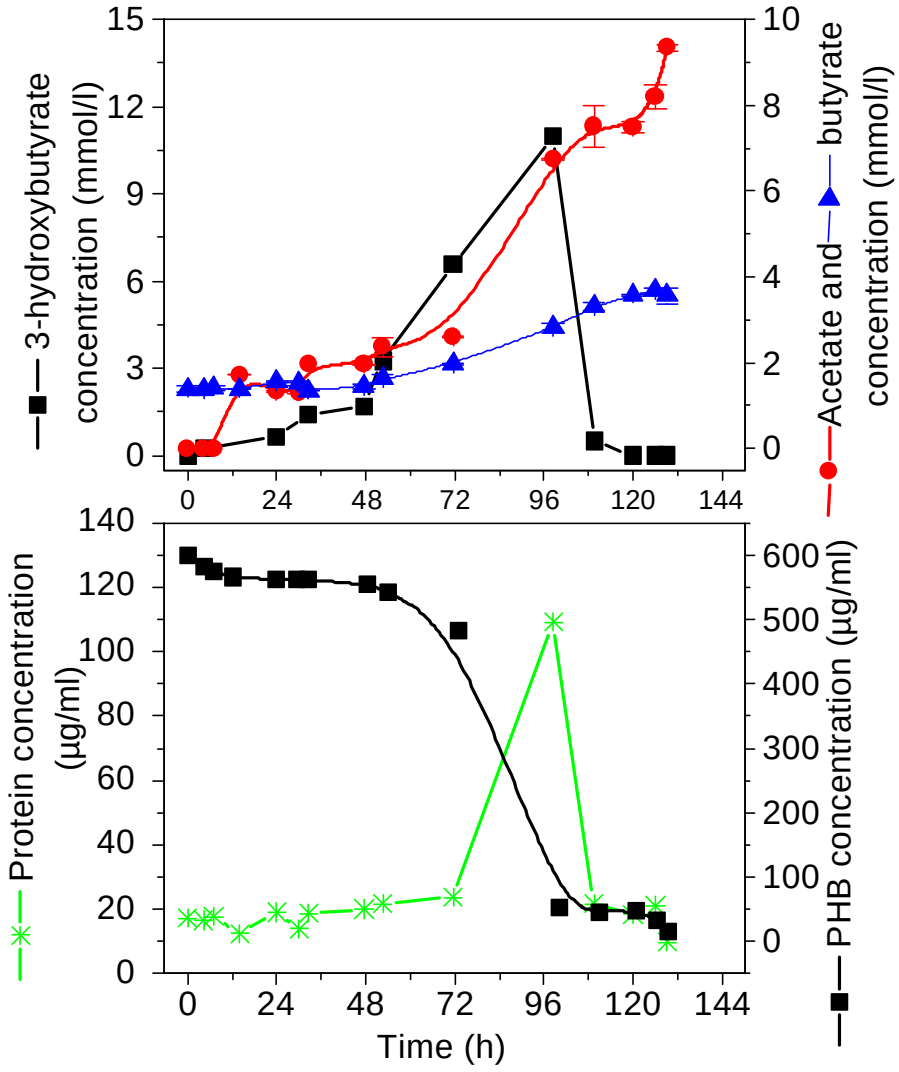


Fig. 4.33. Growth of *C.* sp. nov. strain 5a during the degradation progress of 1 g l^{-1} PHB powder in a bioreactor. (pH: 6.8; T: 37 °C). 3-hydroxybutyrate appearance as well as end product formation (acetate and butyrate). The Biomass is expressed as protein concentration (µg ml^{-1}).

From the information obtained in fig 4.33, it becomes possible to establish a carbon (table 4.12) as well as a hydrogen (table 4.13) balance for the PHB degradation process. This is important to validate the obtained analytic data. For biomass the stoichiometric formula $C_4H_7NO_2$ ($M_{biomass}$ = 101 g mol^{-1}) according to HARDER (1990) was used.

As becomes obvious from table 4.12, about 93 % of the carbon entering the degradation process are recovered. (No CO_2 evolution could be detected during the fermentation progress). This points to a more or less complete PHB degradation process.

Table 4.12. Carbon balance of the degradation of PHB to the end products acetate and butyrate as well as cell growth.

Substance		Concentration		Carbon contents	Carbon quotient Q_c	
		mmol/l	mg/l			
Substrate	PHB-monomer units	11.6	1000	0.558	558	$m_{c\text{-}in}$ 558
Products	Acetate	9.35	561.5	0.407	228.5	$m_{c\text{-}out}$ 521.6
	Butyrate	3.6	324	0.552	179.1	
	Biomass	2.38	240	0.475	114	
ΣQ_c						**93.4 %**

According to GOTTSCHALK (1979) the available H is determined by hypothetically converting all products and biomass through a reaction with water to carbon dioxide and hydrogen as follows:

PHB-monomer: $(C_4H_6O_3)_n + 6_n\ H_2O \rightarrow 4_n\ CO_2 + 18\ H$

Biomass: $C_4H_7NO_2 + 6\ H_2O \rightarrow 4\ CO_2 + 16\ H + NH_3$

Butyrate: $C_4H_8O_2 + 6\ H_2O \rightarrow 4\ CO_2 + 20\ H$

Acetate: $C_2H_4O_2 + 2\ H_2O \rightarrow 2\ CO_2 + 8\ H$

The amount of available hydrogen represents simultaneously the available electrons.

Table 4.13. Hydrogen balance of the degradation of PHB to the end products acetate and butyrate as well as cell growth.

Substance		Concentration:		Balance of available H:	
		mmol/l	mg/l	Available H	Available H (mol/mol)
Substrate	PHB-monomer units	11.6	1000	18	0.210
Products	Acetate	9.35	561.5	8	0.075
	Butyrate	3.6	324	20	0.072
	Biomass	2.38	240	16	0.038
Total	-	-	-	-	0.190
Hydrogen balance: (0.21/0.19)					**1.1**

Both, the carbon balance of 93.4 % as well as the hydrogen balance of 1.1 validate the experimentally obtained data. Since 20.4 % of the total available substrate were utilized for the synthesis of biomass (2.38 mmol/l), only the remaining 79.6 % (9.23 mmol/l) PHB-monomer units are considered for the fermentation balance to acetate and butyrate.

Consequently, this strain degraded the substrate PHB according to the following fermentation equation:

$$5\ \text{–[OCH(CH}_3\text{)CH}_2\text{CO]-} + 6\ H_2O \rightarrow 6\ CH_3COO^- + 2\ CH_3CH_2CH_2COO^- + H_2 + 8H^+$$

Assuming the possible production of 10.5 g dry weight per mol ATP (STOUTHAMER, 1979), the ATP yield would equal 0.75 mol/mol PHB-monomer units degraded. These values are somewhat higher than values obtained for *Ilyobacter polytropus* (STIEB AND SCHINK, 1984) on crotonate and 3-hydroxybutyrate fermentation as well as *Ilyobacter delafieldii* (0.5 to 0.6 mol ATP/mol substrate) (JANSSEN AND HARTFOOT, 1990). However, the same authors stated, that the production of hydrogen results in an increased flow of the more oxidized product, acetate, and thus results in more ATP production.

4.3.5. Alternative analytical determination of growth and PHB degradation during PHB degradation in a bioreactor

Although the method for determination of PHB concentration after SENIOR ET AL. (1972) is very accurate, it is time consuming and requires the work with concentrated sulphuric acid. Therefore, an alternative non tedious analytical procedure for rest polymer as well as cell growth determination had to be established. It must be kept in mind, that PHB degradation in liquid culture applying PHB powder as substrate is a heterogeneous system. Growth of cells which normally is measured as the culture OD_{600nm} interferes with the changing optical density due to PHB degradation which is followed by a decrease in PHB particle size. Thus it was questioned, what influence has cell growth on OD_{600nm}? In order to evaluate the impact of cell growth on the optical density, the organism was grown on the monomer 3-hydroxybutyrate instead of PHB.

The slow growing organism strain 5a has a maximum growth rate of $\mu_{max} = 0{,}047\ h^{-1}$ when grown on the monomer due to the relatively long doubling time of 14.7 h of the organism (fig. 4.34a). Thus, a maximum OD_{600nm} of 0.23 is obtained at the end of the exponential growth phase which is maintained for a maximum of 25 hours.

The elevated optical density values obtained during growth on and degradation of PHB (fig 4.34b) hence are the result of the impact of PHB particles and not due to cell growth. This in turn means, that the measured decrease in optical density determined during the fermentation progress and corrected for the OD_{600nm} values as a result of cell growth (determined through correlation with measured protein content) can be used to monitor the

progress in PHB particle size decrease and hence PHB degradation. This information can thus be used as an indirect indicator for the PHB content. It became obvious from the obtained data, that the decrease in PHB can be determined with sufficient accuracy and reproducibility using OD_{600nm} and protein content data.

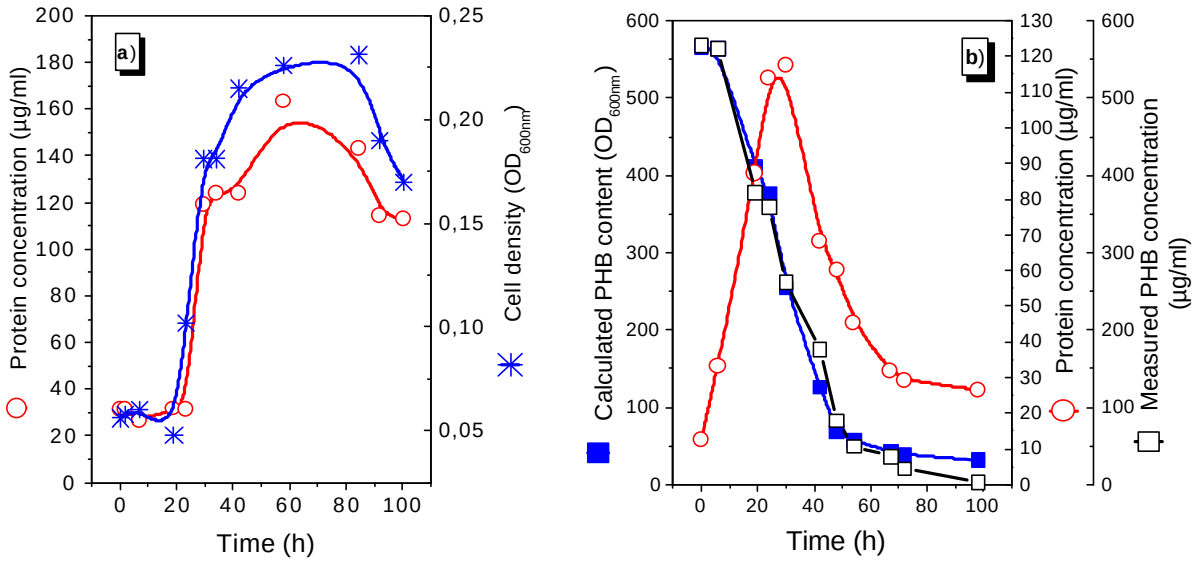


Fig. 4.34. Growth curve of strain 5a grown on (a) 3-hydroxybutyrate and (b) PHB. Growth of strain 5a expressed as protein concentration and PHB degradation elucidated as decrease in OD_{600nm} and through measuring the PHB concentration (SENIOR ET AL. 1972). (MSV-medium supplemented with 0.1 % PHB and 0.1 % yeast extract; pH = 6.8; 37 °C; 150 rpm).

4.3.6. Determination of the factors limiting degradation

Previous experiments as the degradation of PHB and PHBV films on agar plates showed a cessation of degradation after 7 weeks of incubation. The SEM micrographic examination, however, showed no limitations induced by the degradation surface. Thus the question arose, what are the factors influencing PHB degradation with strain 5a? Possible considerations are a change in the culture pH-value or the depletion of nutrients in the medium.

4.3.6.1. Effect of culture pH

Studying the degradation progress of PHB in liquid culture in serum bottles showed that growth and degradation ceased in liquid cultures accompanied by a sudden decrease in culture pH even in strongly buffered media.

Fig. 4.35a shows that growth by strain 5a on PHB in a pH-uncontrolled medium was divided into three phases. First, a lag phase of 20 hours at which the culture pH remained

unchanged at a value of 7. The lag phase was followed by an exponential growth phase during which the pH dropped to 5.5. Finally, the decline phase was reached during which the pH decreased further to values below 5. A maximum weight loss of PHB powder of 207.5 mg equaling only 35 % were obtained. Yet, previous studies with the organism in rich media showed that its pH-range lies between 5 and 7 and that the specific growth rate is almost constant between pH-values of 5.5 to 6.5 (μ_{max} = 3.25 – 3.75, respectively). However, PHB degradation ceased after 70 hours (pH = 6.89) and remained constant thereafter although the organism remained in the exponential growth phase for 30 more hours. During this time the pH decreased constantly to 5.59.

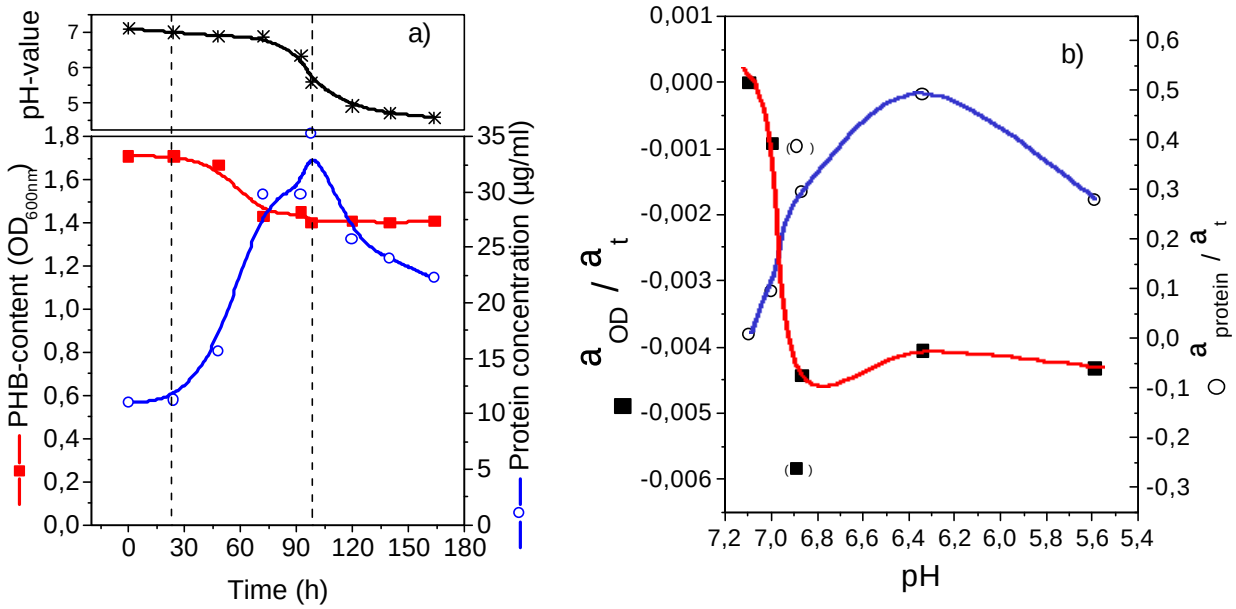


Fig. 4.35. pH dependence of PHB degradation with strain 5a. (MSV-medium containing 1 g l^{-1} PHB and 0.1 % yeast extract; 37 °C; shaken at 150 rpm). On the right the decrease in protein production rate as well as the decrease in PHB degradation rate were plotted against the corresponding pH-vale obtained during growth of strain 5a on PHB.

The increase in protein production rate as well as the decrease in PHB degradation rate were plotted against the corresponding pH-vale obtained during growth of strain 5a on PHB. It became obvious, that PHB degradation only occurred until the pH reached 6.8.

From the degradation experiment in the culture flasks, it could be presumed that pH has a crucial effect on PHB depolymerization. Therefore, the degradation progress of PHB in a pH-controlled bioreactor under defined non-optimal conditions at pH = 5.8 in liquid MSV-culture was studied (Fig. 4.36). Despite the occurrence of some minor cell growth during the first days at a controlled pH of 5.8 (as in the pH uncontrolled serum bottles), PHB was not degraded to a significant amount. Since all conditions were kept constant with the exception

of the culture pH, the lack of growth and degradation was attributed to the unfavorable conditions caused by the low pH-value.

On the other hand, at a controlled pH of 6.8, a short lag phase of 5 hours was followed by PHB degradation which continued exponentially for 50 hours by the end of which about 80 % of the PHB were degraded (fig. 4.36a). Parallel to PHB degradation, the organism grew exponentially as shown by the increasing protein concentration in the culture (fig 4.36b).

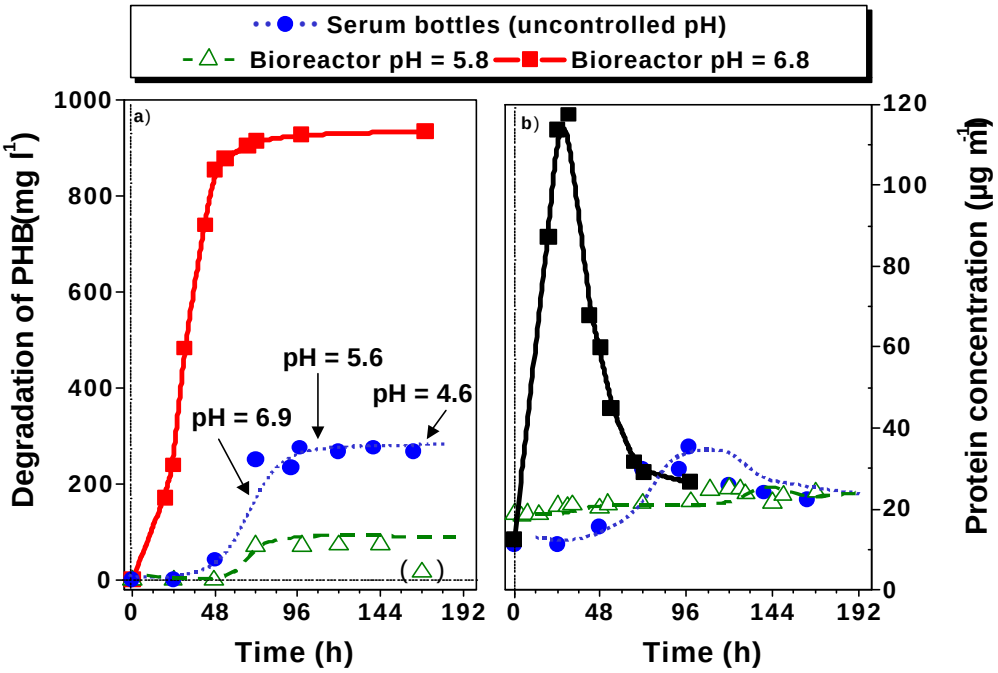


Fig. 4.36. Influence of pH on PHB degradation with strain 5a in a controlled bioreactor. (MSV- medium supplemented with 0.1 % PHB and 0.1 % yeast extract; 37 °C; 150 rpm). **a)** PHB degradation elucidated as decrease in OD_{600nm} and **b)** cell growth determined as protein concentration (µg/ml). pH = 5.8, pH = 6.8 and the pH uncontrolled serum bottles (see Fig. 4.35).

It must be pointed out here, that cell growth in the bioreactor at pH 6.8 was about 3 to 4 fold higher compared to the growth in the pH-uncontrolled serum bottle and under controlled non-optimal pH-conditions (pH = 5.8). Consequently it was presumed that PHB-degradation is limited by the culture pH-value. In complex media growth occurred at pH values between 5.5 and 6.5 with almost constant growth rates. It must consequently be assumed, that intracellular metabolism is not adversely affected by these rather low pH-values. On the other hand, the low pH negatively affected the PHB depolymerization (PHB-

decrease stopped) indicating that the pH value may have an effect on the PHB depolymerase system rather than the intracellular catabolism

4.3.6.2. Effect of surface area

As a second factor influencing the PHB degradation progress, the available surface area was considered, since polyester degradation is considered as a surface process. The degradation progress of PHB in a pH-controlled bioreactor at pH = 6.8 using PHB platelets with two different surface areas (107 cm^2 and 433 cm^2) but the same PHB content (1 g l^{-1}) was studied in liquid MSV medium (fig. 4.37).

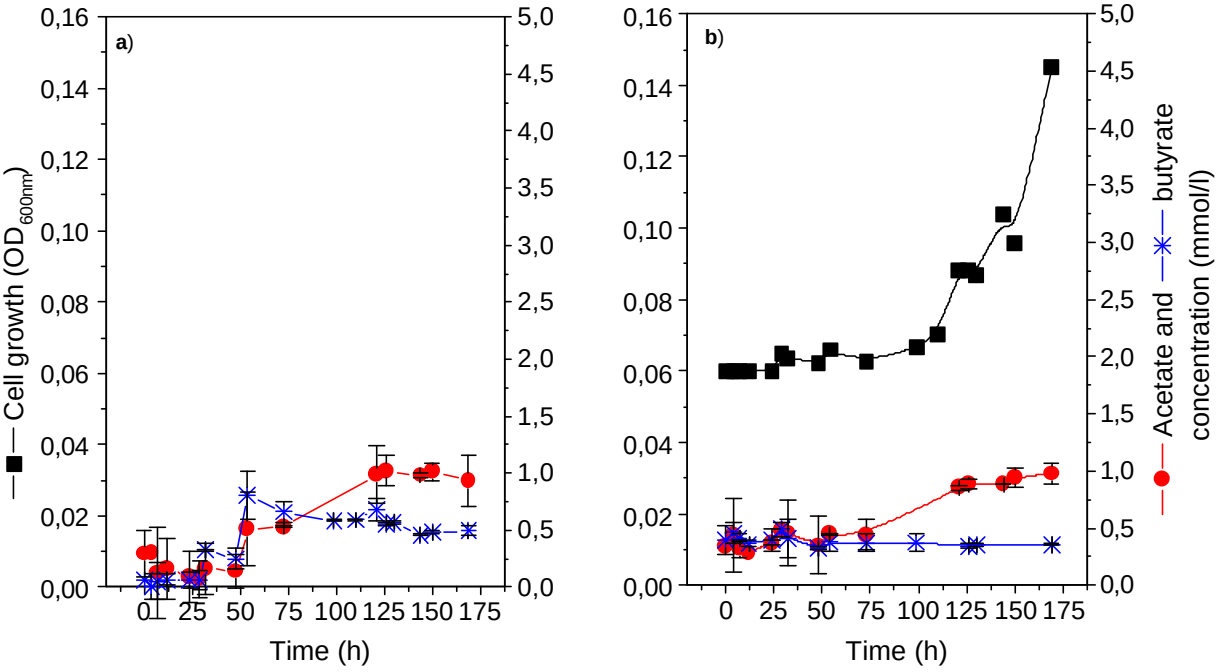


Fig. 4.37. Influence of surface area on PHB degradation with strain 5a in a pH-controlled bioreactor. (MSV-medium with 0,1 % yeast extract, pH 6.8, 37 °C, stirring at 150 rpm, 1 g/l PHB). Reactor **a)** 21 platelets of PHB films with a total surface area of 107cm^2; reactor **b)** 85 platelets of PHB films with a total surface area of 433cm^2.

The results illustrated in Fig. 4.37 show a similar degradation behavior in both reactors, independent of the available surface area. At the end of the fermentation course, the PHB platelets were recovered washed and re-weighed, to determine the PHB weight losses. In reactor a) 67 mg and in reactor b) 66 mg were lost.

If the initial degradation surface of films (107 cm^2) is increased by a factor of 4 no effect is measured and the absolute weight losses as well as degradation rates remain unchanged (table 4.14). This in turn means, that PHB degradation is not primarily depending on the surface area. Calculating the degradation rate per surface area, these values decrease with

increasing surface area. For the degradation of the PHB-powder (table 4.14) a 40-fold increase in the weight loss rates was achieved; however, taking into account the very large surface area of the powder (4.4 $m^2\ g^{-1}$), the degradation rate per surface area drops to about 0.1 $mg \cdot cm^{-2} \cdot week^{-1}$. One possible explanation for this non-linear impact of the available surface area might be the relatively low cell growth resulting in a low enzyme concentration, i.e. the enzyme concentration rather than the available surface area is the limiting factor.

Table 4.14. Influence of the surface area on the degradation of PHB in a controlled bioractor. (MSV-medium, working volume 600 ml, pH 6.8, 37 °C)

Parameter	Films		Powder
	Reactor a)	Reactor b)	see Fig. 4.31
Polyester parameters:			
Number of films	21	85	-
Thickness of films (: m)	160 µm	40 µm	-
Surface area (cm^2)	107	433	26400[a)]
Degradation parameters:			
Weight loss (mg)	67	66	600
Weight loss (%)	(11 %)	(11 %)	(100 %)
Degradation rate ($mg \cdot week^{-1}$)	67 [b)]	66 [b)]	2680[c)]
Degradation rate ($mg \cdot cm^{-2} \cdot week^{-1}$)	0.62 [b)]	0.15 [b)]	0.10 [c)]

a) determined by nitrogen sorption measurements
b) average degradation rate
c) maximum degradation rate

4.3.6.3. Effect of head-space gas composition

Since literature describes the influence of head space gas composition on growth and metabolism of several anaerobic bacteria and clostridia species (HOLDEMANN AND MOORE, 1978), the effect of head space gas composition on the degradation process was investigated.

Studying the effect of head space gas composition, pure N_2-gassing was compared to applying a gas mixture of N_2/CO_2 (80 : 20) on the PHB degradation process using the same fermentation conditions mentioned before.

As shown in fig. 4.38, the head space gas composition has an impact on the duration of the lag phase, which was 4 fold longer (24 h) under a gas mixture of N_2/CO_2 = 80 : 20. Implying that the organism can also grow using CO_2 given that (and as long as) another additional C-precursor is available, PHB depolymerization would presumably not occur, explaining the

lag phase. Yet, care must be taken not to overinterpret this observation due to a lack of additional data and further investigations would be necessary to clarify this situation. As for the degradation process, the same maximum degradation rates (linear part of the degradation curve) were obtained for both head space gas compositions.

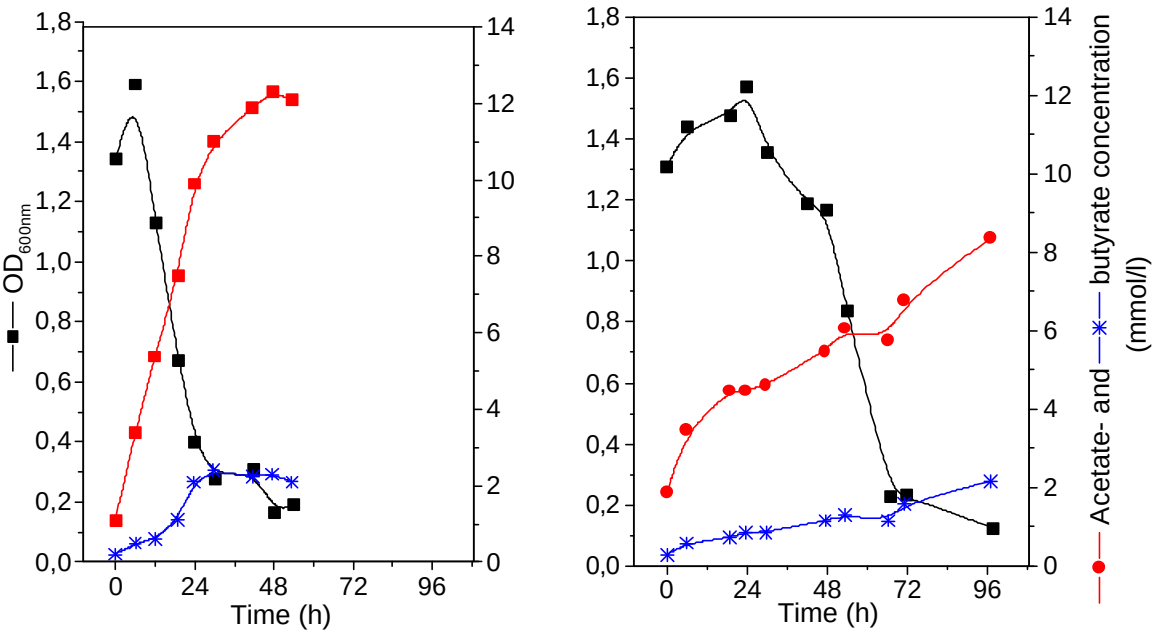


Fig. 4.38. Influence of gas composition on PHB degradation with strain 5a in a controlled bioreactor. (MSV-medium with 0,1 % yeast extract, pH 6.8, 37 °C, stirring at 150 rpm, 1 g/l PHB). PHB degradation expressed as decrease in OD_{600nm} and end product formation by strain 5a. Reactor **a)** gassed with pure N_2 gas; reactor **b)** head space gas composition of N_2/CO_2 = 80 : 20.

In conclusion it can be stated that, the pH-controlled bioreactor turned out to be an appropriate tool to obtain fast and sufficiently reproducible data upon the degradation of the polyester. The effect of the significantly enhanced weight loss per time is caused by the optimization of the growth conditions of the sensitive test strain by pH-control. The increase in available polymer surface area by using powder instead of films enhances the degradation rate but the degradation rate per surface area decreases (compared to using PHB-films), probably due to enzyme limitation. On the other hand, the head space gas composition does not affect the maximum degradation rates obtained, however, an extended degradation period due to a longer lag phase was observed under gassing with N_2/CO_2 = 80 : 20.

4.4. Characterization of the PHB-depolymerizing enzyme system of strain 5a

Having determined the main factors influencing the PHB degradation process with strain 5a, it was aimed in the following part to gain some basic information about the involved enzyme.

The main questions to be answered were: What kind of enzyme is involved? When is the enzyme produced? What are the inducers of enzyme activity? Answering these questions will allow the comparison of the anaerobic enzyme system under investigation to other known aerobic PHB depolymerases and the set up of an adequate enzyme isolation and purification protocol.

4.4.1. Development of a suitable enzyme activity test

In order to isolate, purify and characterize a certain enzyme an appropriate enzyme activity detecting test must be available. The test should be highly selective towards the enzyme of interest, sensitive, fast and reproducible (COOPER, 1980). Several enzyme tests previously applied with aerobic PHB depolymerases are described in literature, however, non of these proved to be suitable for the enzyme under investigation.

Measuring esterase activity with p-nitrophenyl compounds (JENDROSSEK ET AL., 1993B; SCHIRMER ET AL., 1993) was not suitable since the detected enzyme activity did not correlate with the PHB degradation and probably determined unspecific esterases such as proteases.

The drop method for clear zone detection (JENDROSSEK ET AL., 1993A; BRIESE ET AL., 1994) on glass slide covered with a PHB containing medium or directly on agar plates (SCHIRMER ET AL., 1993) also failed probably due to problems with the diffusion of the enzyme into the polyester containing agar medium even after extended incubation periods.

Also trials to detect the enzyme activity using native gels layered onto an activity plate (SCHIRMER ET AL., 1993) was unsuccessful. The negative results obtained with these tests were probably due to low enzyme activity, a general instability of the enzyme if exposed to oxygen or due to problems with enzyme diffusion.

Following the decrease of the OD_{600nm} of a stable PHB-suspension (JENDROSSEK ET AL., 1993A; MÜLLER AND JENDROSSEK, 1993) in a suitable buffer in cuvettes also was not satisfactory.

However, the principle of this test method could be used for the anaerobic enzyme, if the test was run in anaerobic “Hungate”-tubes instead of cuvettes. The test protocol (see chapter 7.12.7.) depends on mixing the sterile filtered culture supernatant with a stable PHB suspension prepared in the reduced medium (normally applied for cultivation) in anaerobic “Hungate”-tubes. After determination of the initial OD_{600nm} of this crude enzyme-PHB-suspension-mixture, it was incubated with constant agitation (150 rpm) at 37 °C for at least 24 hours. The decrease of the optical density of this suspension with time allows tracing of enzyme (production) activity and the calculation of PHB degradation using a standard curve (see appendix, fig. 9.4). To optimize the enzyme test, different substrate concentrations and incubation times as well as two different incubation temperatures were tested.

The results illustrated in fig. 4.39 clearly indicate, that enzyme activity is detectable with this method directly in sterile and cell free culture supernatants pointing to the involvement of an extracellular PHB depolymerizing enzyme. Moreover, enzyme activity was detectable at all tested substrate concentrations. From the average mean curve of data taken at different incubation times, it can roughly be stated that at a constant concentration of the crude enzyme at 37 °C the rate of decrease of OD_{600nm} was linearly dependent on the PHB concentration up to approximately 150 µg ml^{-1}. Higher polymer concentrations were apparently inhibitory and resulted in a decrease of the rate. Similar observations were stated by JENDROSSEK ET AL. (1993B) but not explained. Interesting was the finding that this was not the case at 55 °C, since enzyme activity increased linearly up to 200 µg ml^{-1} and increased thereafter at a lower rate without showing substrate inhibition, at least not at the maximum applied PHB concentration.

The maximum detectable enzyme activity was four folds higher at an incubation temperature of 55 °C compared to 37 °C. This points to the known influence of temperature on enzyme activity since increasing the incubation temperature by 10 °C leads to a two to four fold increase in the enzymatic reaction rate (STELLMACH ET AL., 1988). At 37 °C (lower enzyme activity) the available enzyme concentration (adsorbed on the surface of the particles) is too low to reduce the PHB particle sizes to a measurable effect within the incubation period tested. Elevating the temperature to 55 °C, however, increases the enzyme activity. This means that the same enzyme concentration in relation to the elevated substrate concentration may decrease PHB particle size more rapidly resulting in a measurable decrease in OD_{600nm}.

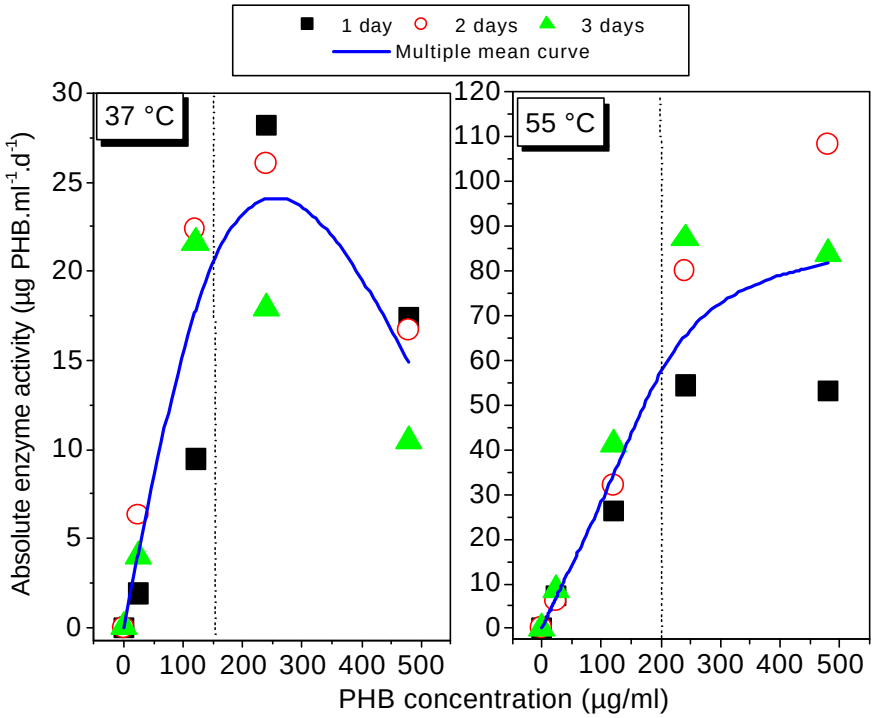


Fig. 4.39. Calculated rate of decrease of OD_{600nm} of a stable PHB suspension as a measure of the detectable enzyme activity. PHB suspension consists of reduced MSV medium with different initial PHB concentrations at 37 and 55 °C. (Notice the different y-axis scaling applied in the two diagrams).

The measured decrease in OD_{600nm} increased with increased incubation periods. However, in the following investigations a PHB concentration of 150 µg/ml (lying within the linear part of the curve), an incubation time of 24 hours and an incubation temperature of 55 °C for enzyme activity detection was chosen to obtain fast and reproducible test results.

4.4.2. Regulation of enzyme production (constitutive or inductive enzyme)

According to SCHLEGEL (1992) most enzyme systems involved in substrate degradation are inductive enzymes. This means that the required enzymes are only produced and secreted in high amounts by the bacterial cell if the specific substrate is present in the direct surrounding. For insoluble substrates, it is generally believed, that under starvation conditions extremely low levels of hydrolytic enzymes, including the target enzyme, would be released into the growth environment. The low levels of substrate hydrolysate which would consequently be generated then enter the cells and induce the synthesis of the enzyme (KOLATTUKUDY, 1984) (see also chapter 3.6.3). The question therefore arose, if the PHB degrading enzyme system is constitutively secreted or induced by the presence of

PHB? And if the enzyme is inducible, which are the substances inducing the enzyme activity?

Fig. 4.40 indicates that the extracellular PHB depolymerase was produced after growth with 3-hydroxybutyrate, lactate and to a lower extent with pyruvate. On the other hand, crotonate does not induce enzyme secretion despite being a good growth substrate. The very low enzyme activities measured for the other substrates (beyond the dotted lines) might be due to a very low basal enzyme secretion or very low enzyme concentrations secreted by the organism in response to starvation, i.e. the absence of the appropriate growth substrate.

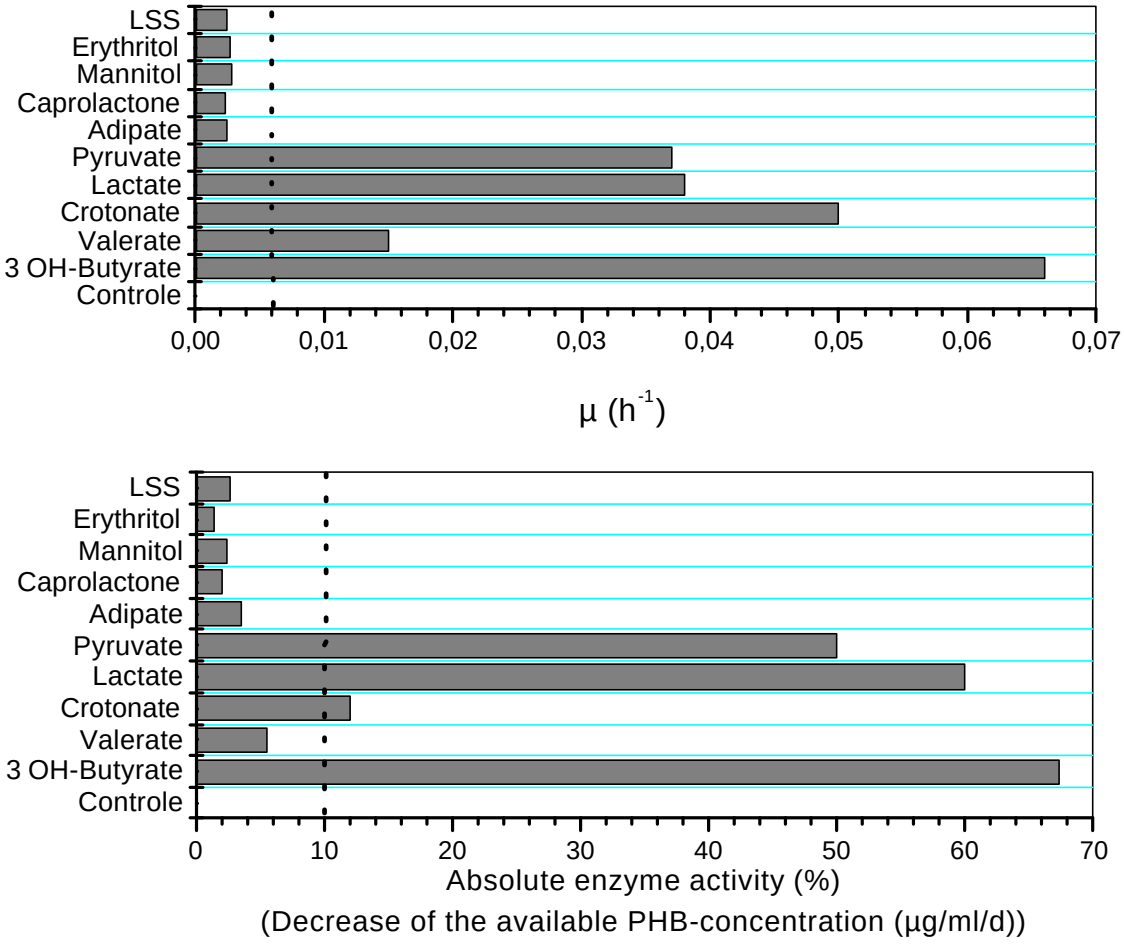


Fig. 4.40. Specific growth rates of strain 5a grown on different growth substrates at 37 °C and PHB depolymerase activities induced by the different carbon sources. (Enzyme activity: decrease in PHB concentration of a stable PHB suspension after 24 h at 55 °C. Growth rate determinations: MSV medium with 0.05 % (w/v) yeast extract and 0.1 % (v/v) of the carbon source inoculated from 10 % (w/v) stock solutions; inoculum: 100 µl from TG medium culture with an initial OD_{600} = 0.5). The dotted lines represent the interpretation limits.

4.4.3. Determination of progress of enzyme activity during fermentation course

The time dependent progress of PHB degradation in a pH controlled bioreactor with strain 5a was investigated in relation to the secretion of the extracellular PHB depolymerizing enzyme. Parallel, the appearance of free 3-hydroxybutyrate (the monomer and end product of PHB depolymerization) as well as cell growth was also monitored (fig. 4.41).

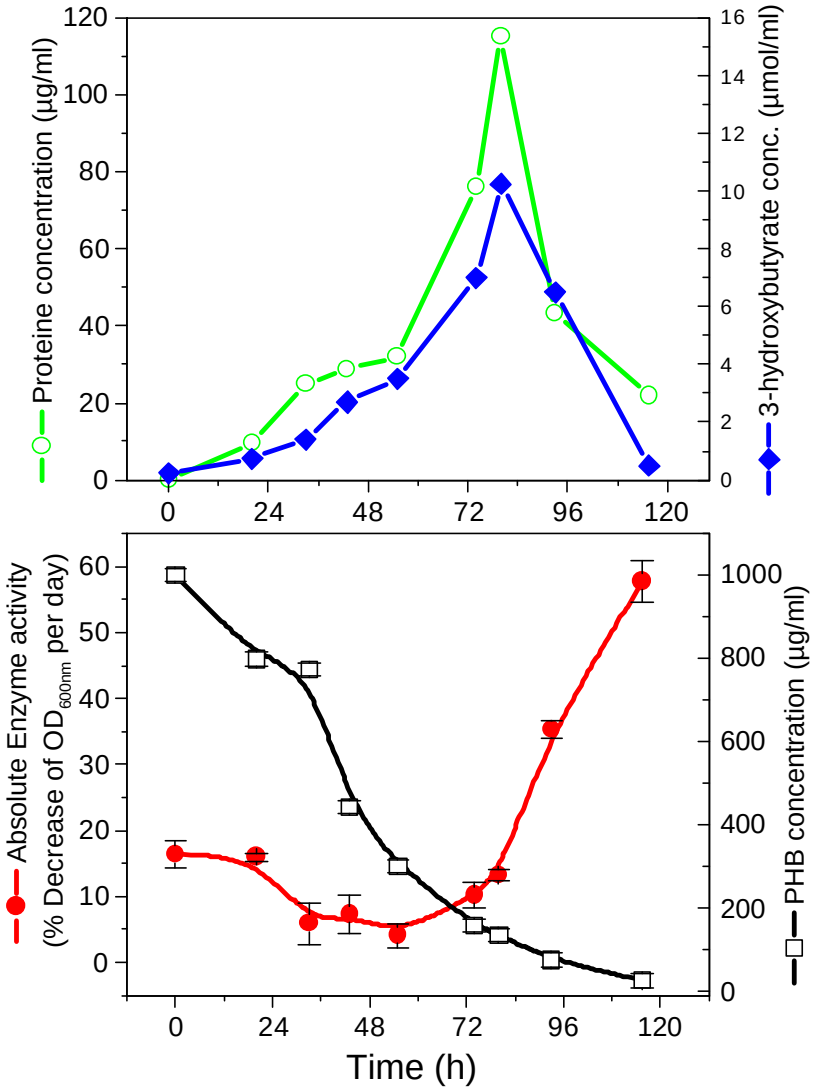


Fig. 4.41. Progress of 3-hydroxybutyrate formation and detectable enzyme activity during the degradation of PHB in a pH-controlled bioreactor. (T: 37 °C, MSV medium with 0.1 % (w/v) yeast extract, pH 6.8, stirred at 150 rpm). Enzyme activity was measured by the decrease in PHB concentration of a stable PHB suspension after 24 h at 55 °C.

Obviously, growth of strain 5a proceeds concomitant with the appearance of 3-hydroxybutyrate (which in turn is the result of PHB depolymerization). Yet, during this phase of active PHB depolymerization, almost no free enzyme activity is detectable in the sterile culture supernatants. However, after depletion of PHB and cessation of monomer

production, free detectable enzyme activity starts to increase. These findings suggest the involvement of an extracellular PHB depolymerizing enzyme which strongly adsorbs to the PHB particles as long as rest polymer is present in the medium. (The possibility of the enzyme being membrane bound was excluded, since cells were never observed to attach to PHB granules throughout the whole fermentation process). Once all the PHB has been degraded and no enzyme specific adsorption surfaces (PHB granules) are available to the enzyme, free enzyme is detectable. The appearance of 3-hydroxybutyrate also ceased with the depletion of PHB from the medium, parallel cell growth stopped and the organism entered the decline phase of growth.

4.4.4. Characterization of the involved PHB-depolymerizing enzyme system

The assumption, that the enzyme binds strongly to the PHB granules during the degradation process was verified by another experiment. A degradation experiment was interrupted while PHB was still present and the enzyme activity was traced in both the pellet, consisting of the rest polymer and cells of the organism, as well as in the culture supernatant. Then, fresh PHB powder was added to the supernatant, removed immediately by centrifugation and the enzyme activity was thereafter determined in the medium again. The data are shown in figure 4.42.

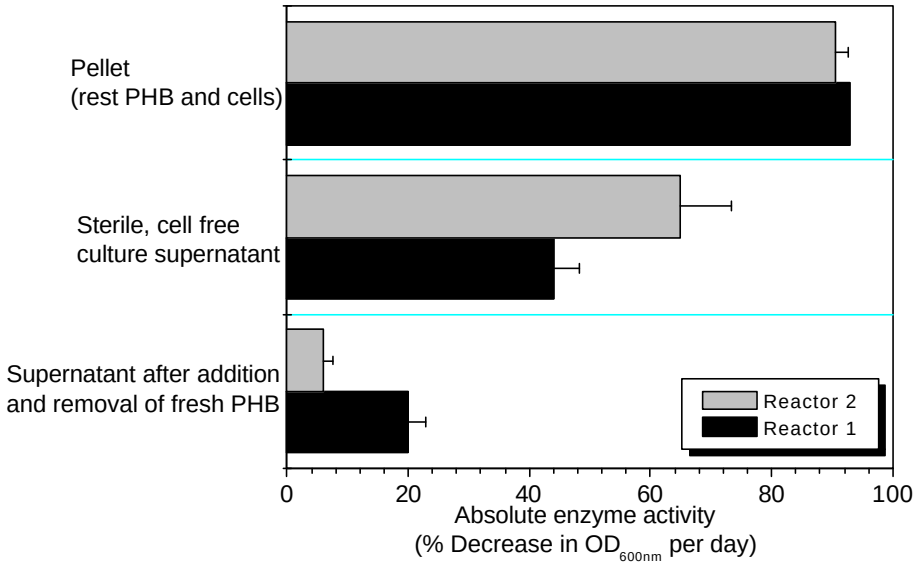


Fig. 4.42. Enzyme activity in the pellet and cell free culture supernatant before and after the addition of 5 mg PHB. (sample 5, time: 74 h, of reactor 1 and 2, see fig 4.43). Enzyme activity was measured by the decrease in PHB concentration of a stable PHB suspension after 24 h at 55 °C.

The major enzyme activity was in fact detected in the washed pellet. The enzyme associated with the pellet is assumed first to depolymerize the rest PHB (of the pellet), and

then consequently be available to bind and depolymerize the freshly added PHB. In contrast to the pellet bound enzyme, only about 40 % of total detectable enzyme activity was found in the cell free culture supernatant. Since the enzyme test was performed at 55 °C, and strain 5a was not able to grow at such an elevated temperature (see table 4.6) no superposition of the results of newly produced enzyme during the activity test with the intact microbial cells was to be expected.

The detectable enzyme activity in the supernatant after adsorption on freshly added PHB decreased drastically. These results lead to the supposition, that the enzyme has a high affinity to the PHB surface. The enzyme, however, does not adsorb completely on PHB despite the presence of excess PHB. Here it can be supposed, that the enzyme structure alters with time, predominately affecting the binding domain, and thus reducing the adsorption ability with time.

To verify this assumption enzymes at three different stages (marked in fig. 4.43 by arrows) during the fermentation progress of PHB degradation were tested for free enzyme activity and the ability of this freely occurring enzyme to bind to native PHB was tested.

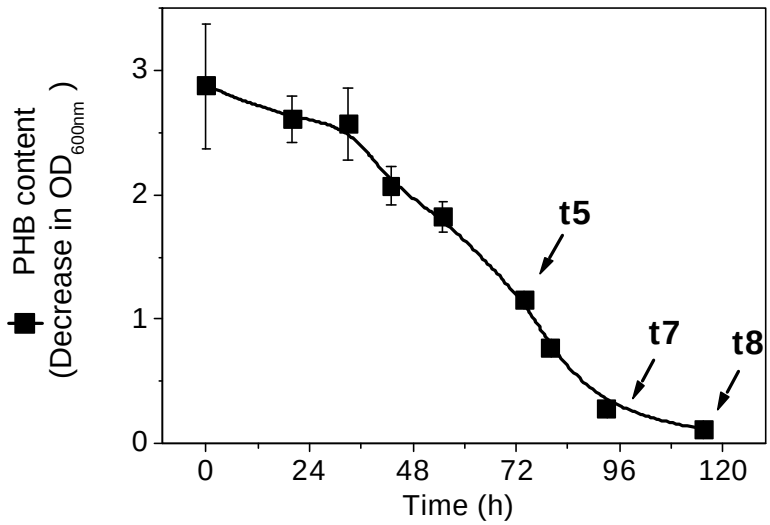


Fig. 4.43. The degradation progress of PHB.
The arrows point to three different samples taken for further investigation and are numbered t5, t7 and t8. (sample of reactor 1 and 2).

As explained previously, the enzyme activity was traced in both the pellet, consisting of the rest polymer and cells of the organism, as well as in the culture supernatant. Then, fresh PHB powder was added to the supernatant, removed immediately by centrifugation and the enzyme activity was thereafter determined in the medium again.

As observed before, the enzyme does not re-adsorb completely despite the presence of excess of PHB (fig. 4.44). The ratio of enzyme activity in supernatant before readsorption on

PHB and the rest activity in the supernatant after adsorption to PHB is not constant and decreases from t5 to t8 by approximately 7 folds. This means, the free enzyme in sample t5 seems to have a higher capability to reabsorb on PHB than enzyme in sample t7 followed by t8. Obviously, the enzyme "ages", i.e. it loses its adsorption ability or affinity to PHB granules.

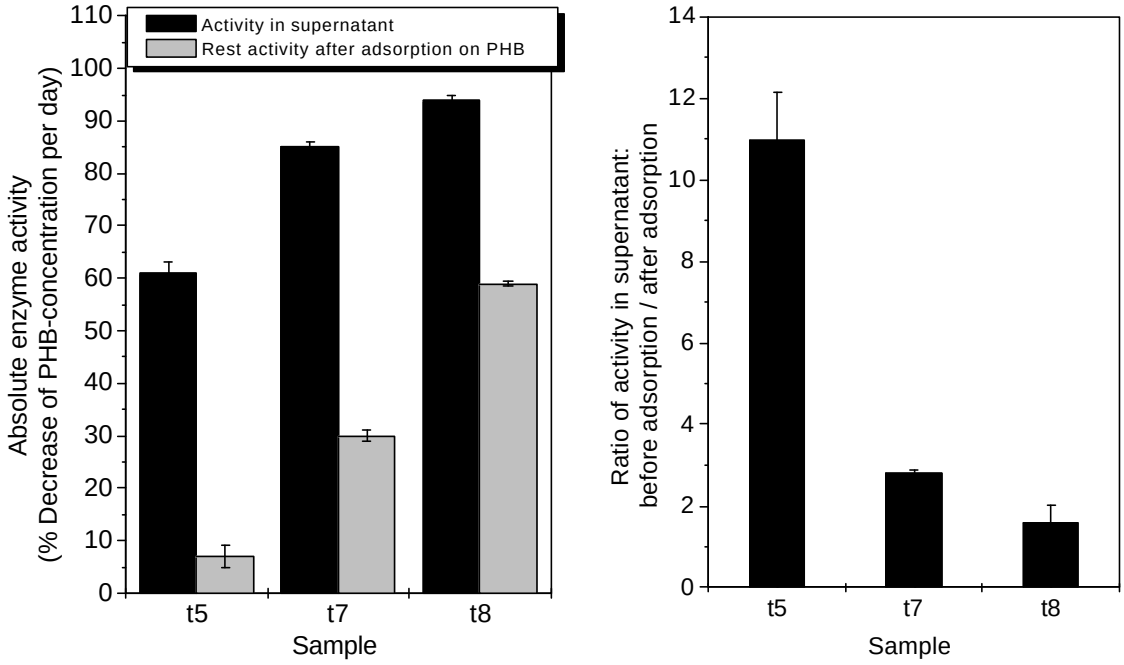


Fig. 4.44. Influence of "enzyme age" on adsorption affinity to fresh PHB powder. Enzyme activity in **a)** cell free culture supernatant taken at different times of fermentation course before and after the addition of 5 mg PHB; **b)** shows the ratio between the measured enzyme activity in the supernatant to the activity in the same sample after the adsorption to PHB powder. (Sample t5, t7 and t8 of reactor 1 and 2; n = 4 parallel tests). (Enzyme activity: decrease in PHB concentration of a stable PHB suspension; 24 h; 55 °C).

4.4.5. Enzyme stability

In order to develop an appropriate enzyme isolation and purification protocol the stability of the enzyme had to be determined (fig. 4.45).

The results shown in fig. 4.45a clearly indicate a general sensitivity of the enzyme towards oxygen. The enzyme activity decreased continuously as a result of exposure to oxygen. On the contrary, in the control tubes (under anaerobic conditions) only a minor decrease in activity over at least 48 hours was observed.

Normally, concentrated enzyme solution can be stored at 0 °C. However, crude enzyme extracts as those used during the following part of the work require storage temperatures

of the enzyme to oxygen as well as freezing and thawing should be prevented (at least for the diluted crude enzyme solutions).

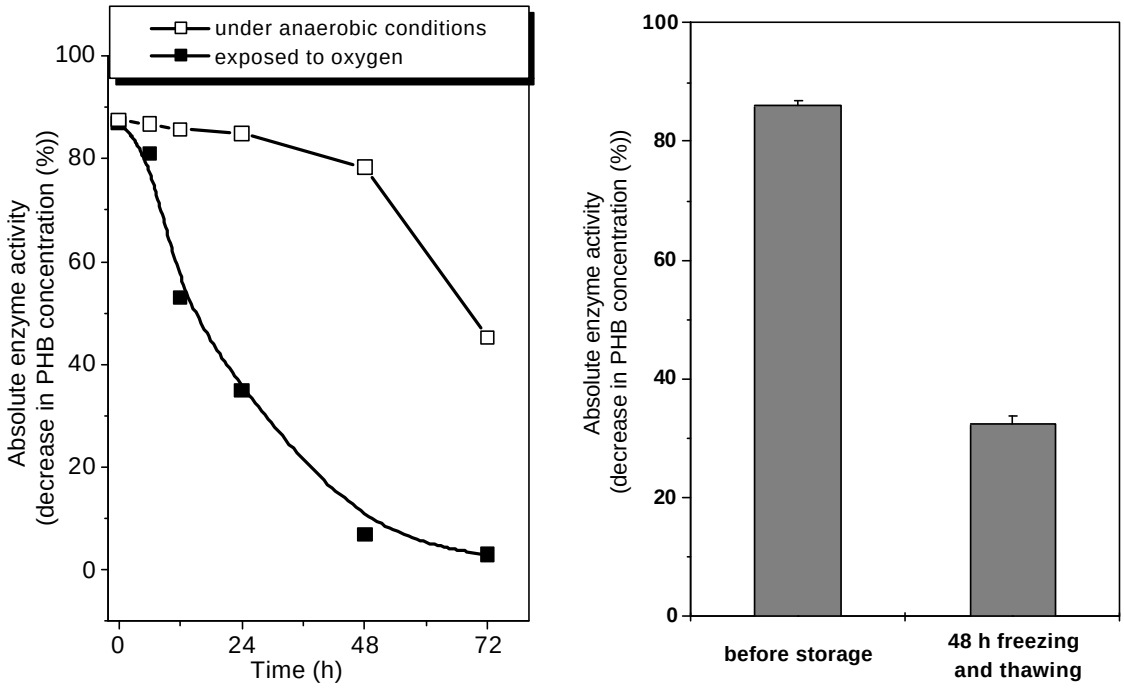


Fig. 4.45. Enzyme stability and activity loss: after **a)** exposure for different periods to oxygen (reduced MSV-medium in "Hungate tubes" (5 ml / 15 ml); shaken with a slope of 60 ° at 150 rpm; RT); **b)** Enzyme activity loss during storage for 48 h at –20 °C and re-thawing. (Sample: F4, R: 1, 2, 3 & 4).

4.4.6. Preliminary enzyme purification studies

In the following, preliminary work to isolate and characterize the enzymatic system, responsible for the PHB depolymerization, is presented.

4.4.6.1. Ultrafiltration

Literature describes ultrafiltration as a effective step for an initial purification since low molecular weight compounds can be separated depending on the cut off of the used membrane (ANDERSSON, 1980). In addition ultrafiltration was successfully applied during purification of aerobic depolymeraes by SCHIRMER ET AL. (1993); JENDROSSEK ET AL. (1993B) AND MÜLLER AND JENDROSSEK (1993). Since it was previously observed, that most of the enzyme activity was associated with the rest polymer present in the culture pellet, it was intended to elute this active enzyme from the rest polymer and concentrate the crude enzyme by ultrafiltration as a first step in the purification process.

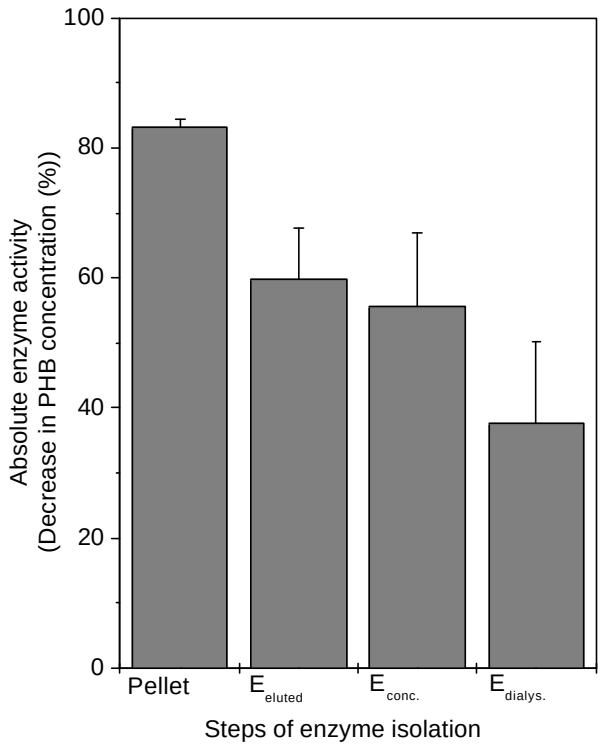


Fig. 4.46. Enzyme stability and activity loss. Elution from PHB-pellet with 50 mM phosphate buffer and 30 % ethanol (pH = 6.8); concentration with an Amicon ultrafiltration unit (volume: 50 ml; filtration area: 47 cm^2; 10 kDa membrane; final volume: 1/8 of the original volume), and subsequent dialysis (8 volumes of 50 mM phosphate buffer). (Sample: F4, pellet of R: 1, 2, 3 & 4; pellet after centrifugation for 30 min, 13 000 rpm).

During elution of the enzyme from the pellet approximately 28 % of the activity was lost and after concentration of the crude enzyme by ultra-filtration a rest activity of 67 % was detected (fig. 4.46). Dialysis resulted in a reduction to 55 % of the original measured enzyme activity. Nevertheless, ultrafiltration and dialysis can be used for an initial concentration of the enzyme sample.

4.4.6.2. Hydrophobic interaction chromatography (HIC)

PHA depolymerases are known to have a pronounced affinity to hydrophobic materials (JENDROSSEK, 1998) and consequently the interaction of the enzyme under test with HIC-materials was investigated. With HIC substances are separated on the basis of their varying strengths of hydrophobic interactions with hydrophobic groups attached to an uncharged matrix. This technique is usually performed with moderately high concentrations of salts in the adsorption buffer (salt promoted adsorption). Elution is achieved by a linear or stepwise decrease in concentration of salts (PHARMACIA, 1997).

Binding of the proteins to the chromatographic material in HIC is dependent on the presence of a certain minimal amount of salt concentration, such as ammonium sulfate, in the buffer.

Thus, the minimal concentration of ammonium sulfate allowing the binding of the target enzyme to phenyl sepharose CL-B4 was determined in a batch test (test tubes, 1ml).

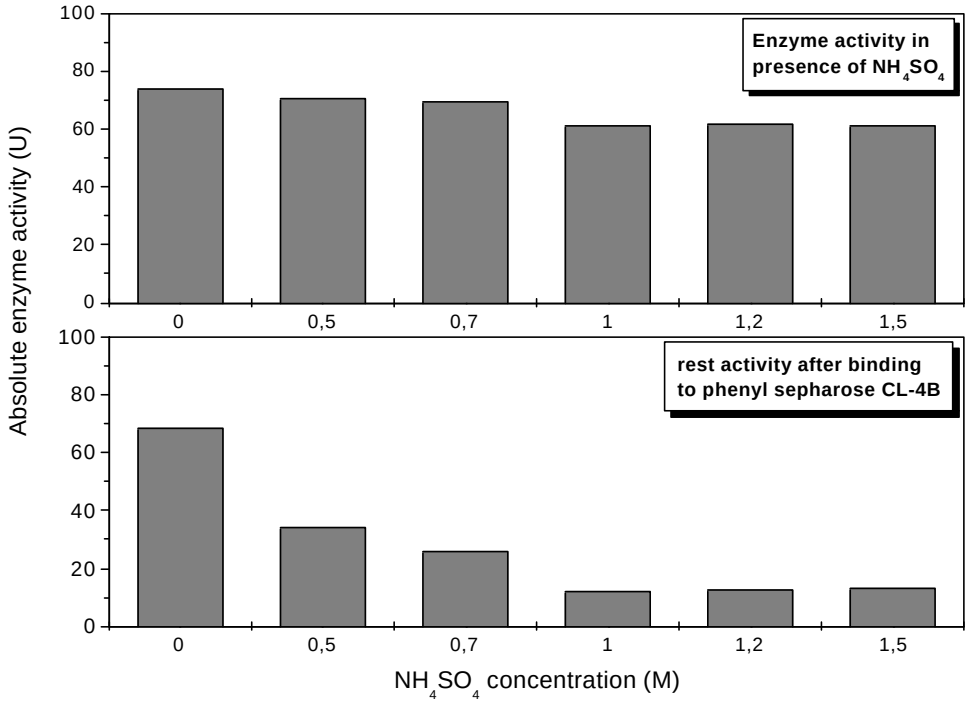


Fig. 4.47. Effect of the applied ammonium sulfate concentration on the enzyme activity and on binding of the enzyme to phenyl sepharose CL-4B. (U = decrease of PHB concentration (µg ml^{-1}) in a stable PHB suspension after 3 days at 55 °C).

The presence of high salt concentrations (fig. 4.47) had only a minimal effect (maximal activity loss of 17 %) on enzyme activity on one hand, however significantly supported the binding of the protein to phenyl sepharose CL-4B. About 50 % of the enzyme were bound to phenyl sepharose on incubation with 0.5 M ammonium sulphate. On further increasing the salt concentration (1 – 1.5 M) an approximately 80 % decrease in enzyme activity was detected. Thus, an ammonium sulphate concentration of 1 M was chosen for further work.

Since the type of immobilised ligand determines primarily the selectivity of the HIC adsorbent, the choice of the ligand was tested by screening experiments using the HIC test kit from Pharmacia (see chapter 7.12.8.3). Two main types were tested: 1) aryl ligands (Phenyl sepharose high performance, Phenyl sepharose 6 fast flow (high), Phenyl sepharose 6 fast flow (low)) which show a mixed mode behaviour, where both aromatic and hydrophobic interactions as well as lack of charges play simultaneous roles. 2) straight alkyl chains (butyl and octyl sepharose (Butyl sepharose 4 fast flow, Octyl sepharose 4 fast flow)) which show a pure hydrophobic character (PHARMACIA, 1997).

A 20 mM phosphate buffer with 1 M ammonium sulfate (pH = 6.8) and 0.5 ml of a concentrated (ultrafiltration, 30 kDa cut off) active enzyme sample were applied. Eluting the active crude enzyme sample from the various HIC materials using a descending gradient of salt concentration (from 1 M to 0 M ammonium sulfate; using 20 mM phosphate buffer (pH = 6.8), and optionally with 30 % isopropanol) resulted in a peak detected by the UV-detector. (The active enzyme was not adversely affected by the presence of 30 % isopropanol since activity decreased by approximately 8 % in presence of the solvent). However, neither the protein peak (representing separated impurities) nor the various protein bands obtained on SDS-PAGE did correlate with enzyme activity (data not shown). Trying to separate the enzyme with gel filtration resulted in a similar situation (data not shown). Obviously, the strength of interaction between the enzyme and the chromatographic material leads to difficulties in the elution of the bound target proteins. Probably the strong binding capacity of the enzyme - such as the strong adsorption to PHB previously observed - plays a key role. Alternative attempts for an enzyme purification are described in chapter 4.4.8.

4.4.7. Enzyme characterization by preliminary gel electrophoretic investigations

4.4.7.1. Native gel electrophoresis for activity testing

As mentioned before (see 4.4.1.), trials to detect enzyme activity using native gels layered onto a turbid, gel containing PHB-powder (SCHIRMER ET AL., 1993) were not successful. The addition of PHB (0.1 %) directly into the native gel matrix as a substrate for PHB depolymerase activity was tested. It had to be clarified, if the presence of the polymer affects the electrophoretic behavior/separation of the protein bands. This was done by comparing the stained native gel with stained SDS-PAGE in presence and absence of PHB (fig. 4.48).

Fig. 4.48 a) and b) showed no differences in the separation behavior of the protein bands although bands appeared less sharp. Therefore, PHB can be incorporated into the chromatography gel for activity testing without adversely affecting the separation process.

Interesting was the finding, that the same enzyme probes were separated on SDS-PAGE into a huge number of bands. On the other hand, the same samples resulted in two separate bands of a high molecular weight (above 94 kDa) on native gels (Fig. 4.49 a, b as indicated by the arrows). This in turn implies, that these protein bands which probably represent the enzyme are not monomers, as would have been expected for a PHB depolymerase, but are oligomers consisting of several subunits which are separated on SDS-PAGE.

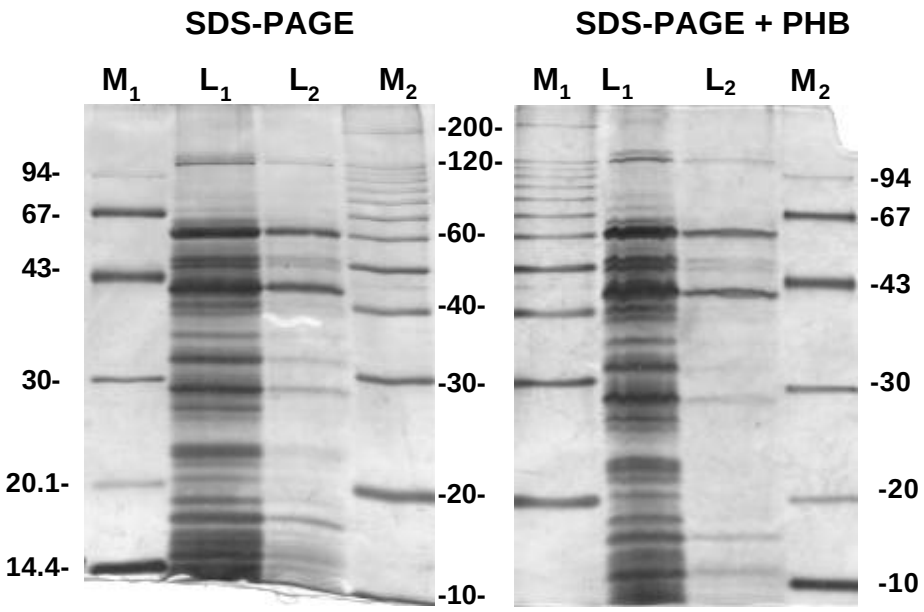


Fig. 4.48. Comparison of SDS-PAGE (a) without PHB and (b) with 0.1 % PHB directly incorporated into the gel matrix. Lanes: **M1** low molecular weight standard (kDa), **L_1, L_2** two different concentrations of an concentrated active enzyme sample (ultrafiltered, cut off 30 kDa), **M2** 10 kDa ladder marker.

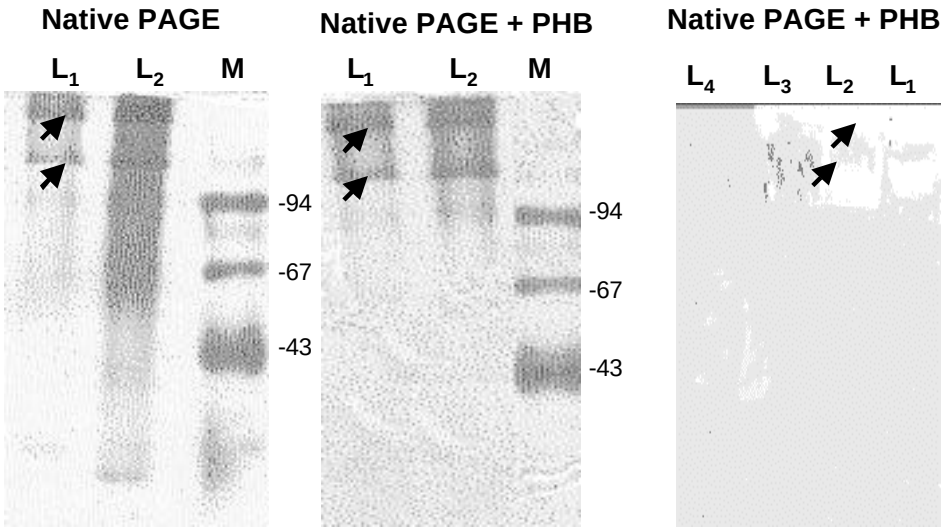


Fig. 4.49. Comparison of native gel electrophoresis (5 %) (a) without and (b) with 0.1 % PHB directly incorporated into the gel matrix. c) Activity gel. Arrows point to the zones of clearance on the activity testing native gel after incubation for 48 hours at 55 °C which correlate in their position with the marked uppermost bands (arrows) in gel c and d. (Lanes: **M** low molecular weight standard (kDa), **L_1, L_2** two different concentrations of an concentrated active enzyme sample (ultrafiltered, cut off 30 kDa), **L3** moderately active enzyme sample, **L4** inactive enzyme sample).

After running two identical PHB-supplemented native gels with an active enzyme sample, one gel was silver stained to determine the position of the separated protein bands (fig. 4.49 b) and its replica was immersed in reduced MSV medium and incubated anaerobically at 55 °C for at least 48 hours. This activity native gel presented in fig. 4.49 c clearly shows zones of clearance near the upper border of the gel. The position of the clear zone correlates with the two upper most protein bands in the stained counterpart (fig. 4.49 b) which did not diffuse significantly into the gel. These two bands show a molecular weight above 94 kDa as marked by the standard. This in turn clearly proves that the target protein is an enzyme with a high molecular weight.

4.4.8. Enzyme purification and characterization

Knowing to have a enzyme protein of more than 100 kDa, a cell free fermentation broth containing active crude enzyme was diafiltrated using a membrane with a cut off of 100 kDa (Omega^{TM} unit, membrane: modified polysulfon, ∅ = 60 mm; volume: 150 ml; Pall-GelmanSciences), thereby reducing the initial volume by 47 folds. In course of the concentration process the absolute enzyme activity of the initial crude enzyme sample (U = 31.5) did not increase proportional to the absolute activity after the enzyme concentration (fig. 4.50), which is the sum of the activity in the concentrate plus that of the washing steps (U = 176).

Two possible reasons were considered. First, the enzyme was damaged during the diafiltration procedure and therefore lost activity. The second reason could be the non specific adsorption of the enzyme to the ultrafiltration devices, especially the membrane. Since the high adsorption capability of the enzyme was noticed before, the ultrafiltration membrane used for the concentration of the crude enzyme solution was treated twice with 20 ml and a third time with 50 ml of 20 mM phosphate buffer containing 30 % ethanol as an eluent (pH = 6.8) for 10 minutes under continuous shaking to remove probably adsorbed enzyme.

Fig. 4.50 clearly shows that most of the active enzyme is present in the membrane wash eluent (MW1 and MW2) and only partly exists freely in the concentrated enzyme solution as demonstrated by the absolute and specific enzyme activities detected during each working step. This behavior underlines the previously observed high tendency of the enzyme to adsorb to hydrophobic surfaces.

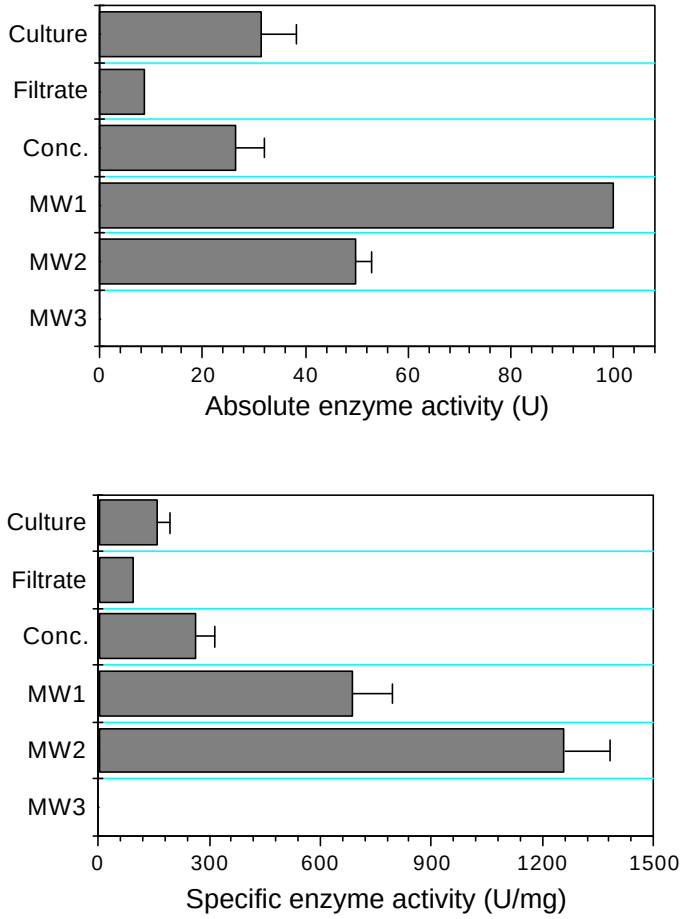


Fig. 4.50. Comparison of the absolute and specific enzyme activities detected in the cell free culture broth (culture), the filtrate, the concentrated enzyme after ultrafiltration (conc.) and the three subsequent membrane washing steps (MW1 – MW2) using 20 mM phosphate buffer with 30 % ethanol (pH = 6.8). For MW3 50 ml 20 mM phosphate buffer with 30 % ethanol (pH = 6.8) were used. (U= decrease in PHB concentration (µg/ml) of a stable PHB suspension after incubation for 3 days at 55 °C).

4.4.8.1. Purity control

The purity of the enzyme in the cell free culture broth and the concentrate was verified using a 5 – 12.5 % PhastGel^{TM} native Gradient (PharmaciaBiotech).

As shown in fig. 4.51, the concentrated enzyme solution consisted of only two high molecular weight bands of more than 670 kDa and about 440 kDa. These two bands were suspected to be the two bands previously observed in fig. 4.48. Considering the distance of the first band relative to the marker protein band of 669 kDa, it might be possible, that the first protein band represents the dimer of the second band.

However, further investigations are necessary to clarify this assumption. It would be interesting to investigate the composition of the oligomers and to clarify whether or not they contain identical or different subunits. However, these questions were not followed up further due to time limitation.

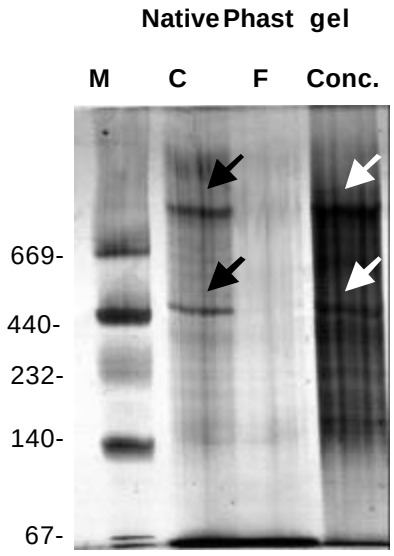


Fig. 4.51. Controle of the enzyme purity using a 5 – 12.5 % PhastGel™ native Gradient (PharmaciaBiotech). The gel was run using the PhastSystem™ with HMW calibration kit protein standards (M; molecular weights in kDa). Lanes: (C) cell free culture broth; (F) the filtrate; and (conc.) the concentrated enzyme after ultrafiltration.

4.4.8.2. Activity detection of the isolated protein bands

To proof which of the two high molecular weight protein bands represents the active enzyme, the concentrated enzyme sample was applied on two subsequent lanes on a native polyacrylamide gel (5 %) containing 0.1 % PHB as a substrate for enzyme activity testing. After electrophotetic treatment the first two lanes were separated, silver stained and are shown in fig 4.52 (left).

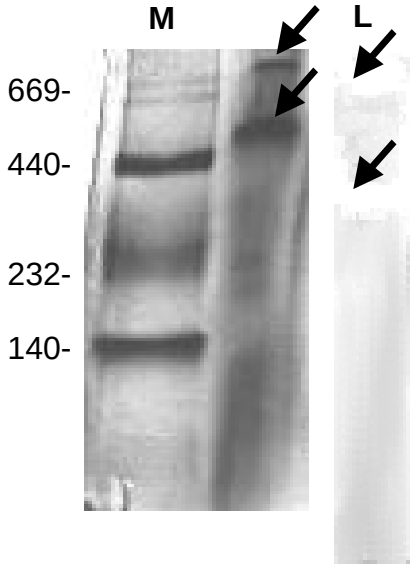


Fig 4.52. Native polyacrylamide gel electrophoresis for the detection of the PHB active protein band. Left: Silver stained native gel containing 0.1 % PHB. Lane M: High molecular weight calibration kit (Pharmacia); Lane L: concentrated enzyme probe. Right: L: concentrated enzyme probe incubated in reduced MSV medium at 55 °C for 24 hours. Arrows point to the developed clear zones. Differences in position of the clear zones and the stained protein bands are attributed to shrinkage of the left gel during the staining and preservation procedure.

The arrows point to the position of the two protein bands. The third lane containing the non stained, active enzyme was incubated in reduced MSV medium at 55 °C for 24 hours until

clear zones developed on the gel as seen in fig. 4.52 (right). The arrows point to the clear zones which correspond in their position the two stained protein bands on the stained gel.

This means that both isolated protein complexes exhibit enzymatic activity towards PHB. This in turn implies that either the second protein complex with a molecular weight of over 440 kDa is a subunit of the bigger protein complex or a second distinct PHB depolymerase. At this point, however, it is not possible to come to a final decision.

4.4.8.3. Total protein balance

Table 15 summarizes the details of the enzyme recovery process. The low protein recovery of 68 % (for the total protein) and 12.8 % (for the enzyme) may be explained by the tendency of the enzyme to bind to surfaces. However, the specific activity was enriched by a total of about 14 folds (concentrate + membrane wash 1 + membrane wash 2). In fact, the purification yielded a protein which was pure as demonstrated by gel electrophoresis (fig. 4.51 and 4.52).

Table. 4.15. Total protein balance of the enzyme concentration and purification procedure.

Sample	Volume (ml)	Protein concentration (µg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Protein recovery (%)	Activity enrich-ment
Fermentation broth	1700	36.2	61.54	9784.9	159	100	1
Ultra/dia-filtrate	2464	13.8	34.00	3230.3	95	55	-
Concentrate	36	19.9	7.16	1877.0	262	12	1.6
MW1	20	27.2	0.55	357.5	686	0.9	4.3
MW 2	20	9.3	0.19	236.0	1269	0.3	8
MW 3	50	0	0	0	-	0	-
Total protein balance			**41. 9**			**68**	
Enzyme balance			**7.9**			**12.8**	

This table, however, indicates that the protein concentration is rather low and even the concentrated enzyme samples are still to diluted for preservation. Protein solutions with low concentrations (≤ 50 µg/ml) are unstable and are not suitable to be stored (SUELTER, 1990). Yet, the enzyme was not concentrated further in order to prevent further protein loss by adsorption to working devices.

4.4.8.4. Temperature optimum

Results of enzyme activity with cell free culture filtrates showed a four fold higher absolute enzyme activity at 55 °C compared to 37 °C over a test period of three days (see chapter 4.4.1., fig. 4.39). For the purified concentrated enzyme(s) two distinct temperature optima were detected. For a activity test period of three days a small maximum could be observed at 37 °C and a second but significantly higher maximum at 55°C was found. The higher activity at 55 °C compared to 37 °C corresponds with the findings from the culture filtrate (see 4.4.1.). However, if the time of the activity test is extended to 7 days a different course of the PHB depolymerization is found. In this case the first maximum at 37 °C is higher than that at 55 °C (fig. 4.53).

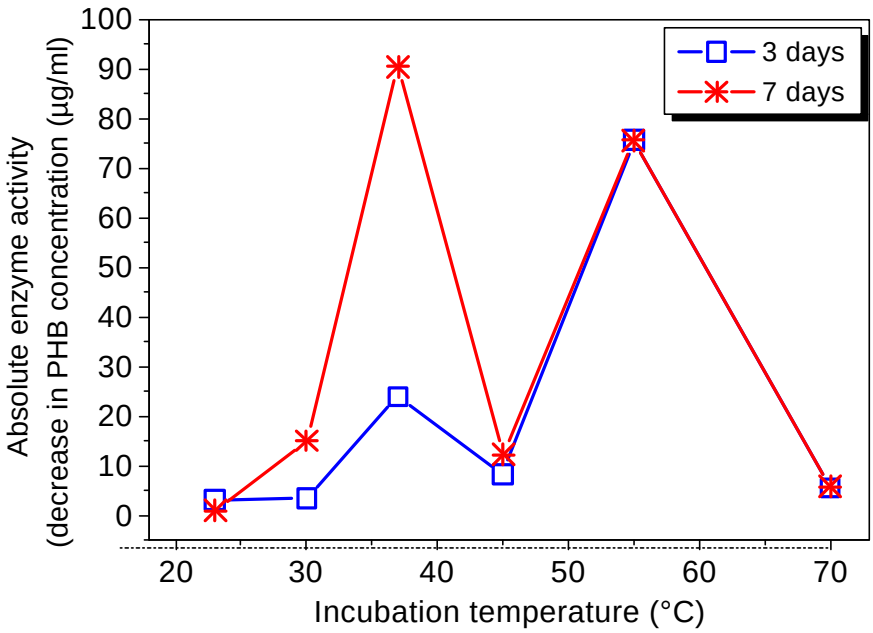


Fig. 4.53. Effect of temperature on the enzyme activity of the purified as well as the concentrated enzyme sample active towards PHB.

The two maxima give an indication on the presence of two distinct enzymes with different temperature characteristics. For 3 days activity testing, the enzyme which was stable under an elevated temperature causes more PHB degradation due to the higher activity present in the solution. However, if the activity test is stopped after 7 days the reduction in PHB turbidity is higher for the mesophilic system, due to the extended test period. In contrast, for the enzyme with the 55 °C maximum, no further PHB degradation is observed within the last 4 days of incubation. Probably here the higher test temperature causes a more rapid deactivation of the enzyme in the solution.

4.4.8.5. pH-Optimum

For the determination of the pH optimum different buffer systems were used. For a pH range of 4 to 7.5 a 0.1 M citrate buffer was applied. The pH values of 8 to 9 were prepared with 0.1 M glycine buffer and the remaining two pH values (10 and 11) were obtained using a 0.05 M borate buffer. Keeping in mind the possible existence of two distinct enzyme complexes, it was aimed to determine the pH optimum for the PHB depolymerase at the two different temperatures 37 and 55 °C.

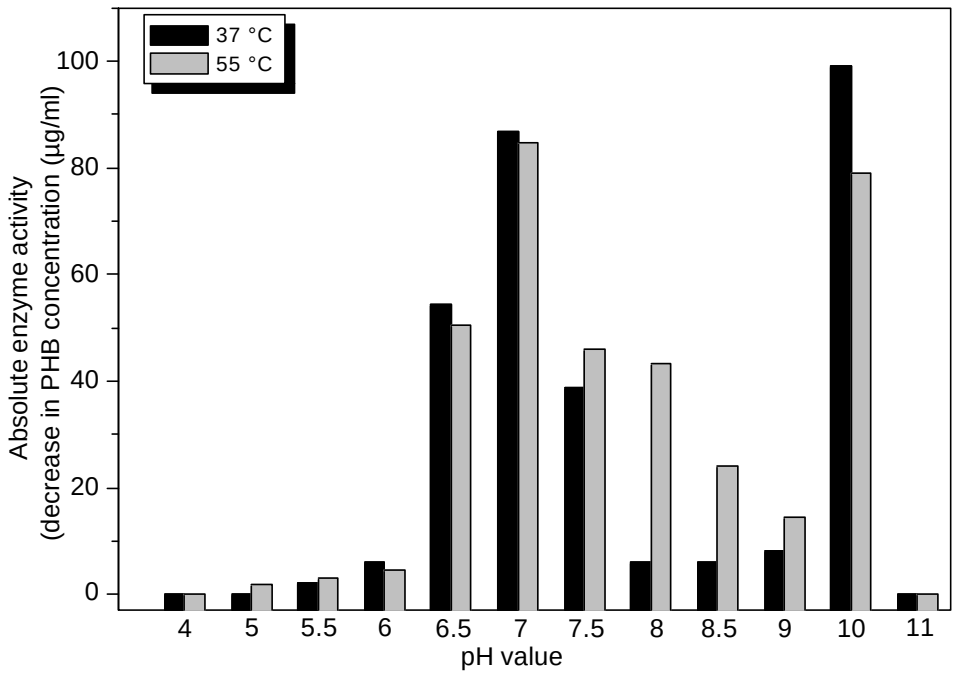


Fig. 4.54. **Effect of different pH values on the purified PHB depolymerizing enzymes.** Enzyme activity was determined by the decrease of PHB concentration of a stable PHB suspension measured at two different incubation temperatures (37 and 55 °C) after 7 days.

As expected from previous degradation experiments a pH optimum for PHB depolymerization at pH = 7 at both tested incubation temperatures were detected (fig. 4.54). Yet, a second pH optimum appeared at a pH value of 10. This unexpected finding once again points to the possible existence of two distinct PHB depolymerizing enzyme complexes. The first one with a temperature optimum of 37 °C and a maximum enzyme activity at a pH value of 10; and the second one with a temperature optimum of 55 °C and a maximum enzyme activity measured at a pH value of 7. However, further investigations are necessary to confirm these preliminary interpretations.

4.4.8.6. Kinetic aspects of PHB hydrolysis

As shown before (section 4.4.1.; fig. 4.39) at a constant enzyme concentration, the rate of PHB depolymerization is linearly depending on the PHB concentration over a narrow range

of substrate (up to 150 or 200 µg/ml at 37 and 55 °C, respectively). Then, at higher PHB concentrations, the degradation rates level to a constant value when the tests are run at 55 °C while at 37 °C the rate of depolymerization decreases again.

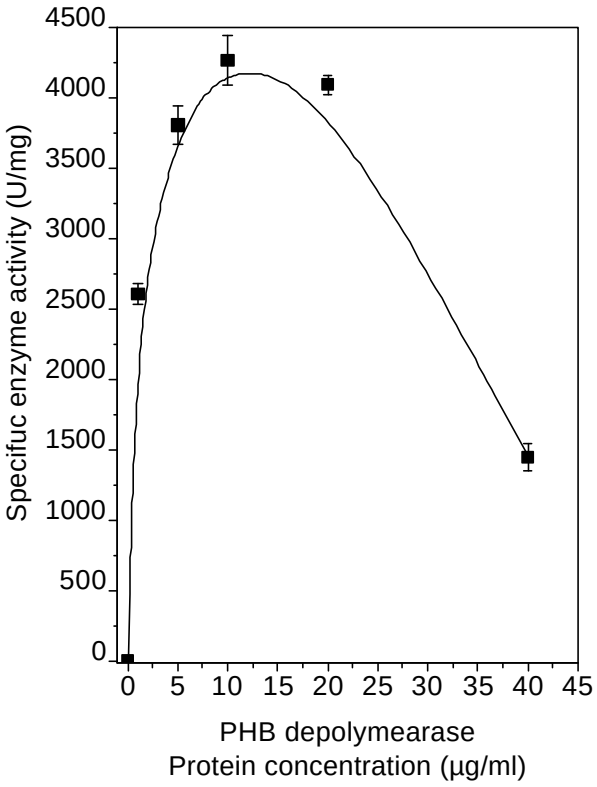


Fig. 4.55. Dependence of PHB depolymerase activity (U = Decrease of PHB in µg/d) on the enzyme concentration. PHB depolymerase activity was measured as decrease in PHB concentration at 55 °C in 4 ml reduced MSV medium containing 300 µg ml^{-1} PHB and various amounts of the purified enzyme.

A similar behavior was observed when the PHB concentration was kept constant at 300 µg ml^{-1} and the enzyme concentration was varied. The rate of hydrolysis at 55 °C was linearly dependent on the amount of the enzyme up to a concentration of purified PHB depolymerase of 5 µg ml^{-1} and then decreased again when higher enzyme concentrations were applied (fig. 4.55).

Similar observations for aerobic PHB depolymerases by a self-blocking inhibition, owing to a condense adsorption of a dual functional PHB depolymerase consisting of catalytic and substrate binding domains were previously explained (MUKAI ET AL., 1993A & B; DOI ET AL., 1994; KASUYA ET AL.,1999) (fig. 4.55).

Applying this model to the conditions of the anaerobic depolymerase(s) it may be concluded that at low concentrations below 5 µg ml^{-1} of the PHB depolymerase the majority of catalytic domains of adsorbed enzymes are able to hydrolyze PHB chains on the surface. On the

contrary, at high concentrations of the enzyme (≥ 10 µg ml^{-1}) the majority of catalytic domains are not accessible to PHB chains due to a condense coverage of the polyester surface with the substrate-binding domains (fig 4.56).

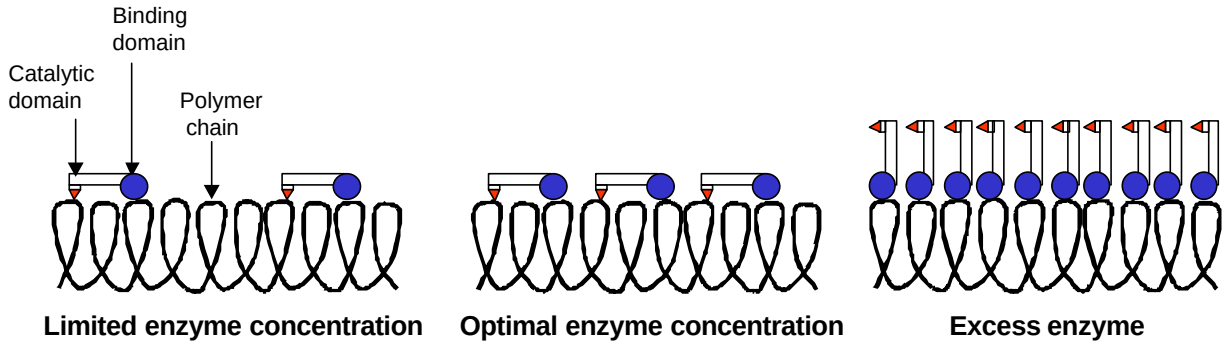


Fig. 4.56. Schematic representation of the enzymatic cleavage PHB by PHB depolymerase. Optimal enzyme activity is dependant on enzyme concentration (after DOI ET AL., 1994).

It must be pointed out here, that such observations were first made by MUKAI AND COWORKERS (1993) using different PHB depolymerases from *Pseudomonas picketti*, *Comamonas testosteroni* and *Aalcaligenes faecalis* having a molecular weight of 40, 49 and 96 kDa, respectively. DOI ET AL. (1994) followed up the investigation including additionally the depolymerases of *Pseudomonas lemoignei* A and B with molecular weights of 55 and 59 kDa. The enzyme used in the present study, however, has a much higher estimated molecular weight of approximately 700 kDa. Calculating its radius after the equation of TONFORD (1961):

$$R = 0.72\ M^{1/3}$$

R = The radius of a globular protein (Å)

M = Molecular weight [g mol^{-1}]

results in 63.9 Å (= 6.4 nm) and hence a cross area of 128.4 nm^2 per enzyme molecule compared to a cross area of 22.1 nm^2 for an enzyme with an average molecular weight of 50 kDa.

It must be kept in mind, that an inhibition was observed at a protein concentration of 10 µg ml^{-1} using 300 µg ml^{-1} of PHB (average particle size: 1 µm; surface area: 4.4 $m^2\ g^{-1}$) in the enzyme test. DOI ET AL. (1994), having an enzyme with a molecular weight of 50 kDa, postulated that 0.1 µg of his enzyme would cover a surface area of 2 cm^2. With the present enzyme (700 kDa) a surface area of 11.6 cm^2 would be covered by 0.1 µg enzyme taking

the differences in size into consideration. Having in the present enzyme test an available surface area of 1320 cm^2 (300 µg PHB) then 11 µg of this protein would cover the entire surface. This is in accordance with the inhibitory concentration of enzyme measured in the present test, and hence is explainable with the model of DOI ET AL. (1994).

4.4.9. Discussion

Studies with the isolated *Clostridium* sp. nov. strain 5a showed that it can grow fermentatively with exogenous PHB. Monomeric 3-hydroxybutyrate was detectable during the fermentation progress. It is the end product of the depolymerase activity. However, from the measurements presented here, it cannot be concluded if oligomers such as dimers and trimers appear as intermediate depolymerase products. 3-hydroxybutyrate is intracellularly fermented to acetate and butyrate as well as energy which in turn is used for growth. The carbon balance experiments recovered 93 % of the added carbon in form of PHB and the hydrogen balance revealed a value of 1.1 validating the experimental data and pointing to a more or less complete metabolization of the polyester.

Generally, it can be concluded that bacteria such as strain 5a could well be the primary fermenting actors in complete methanogenic PHB degradation. The limited metabolic capacity of this strain and its ability to grow on PHB as exclusive carbon source suggest that the organism may be specialized to degrade exogenous PHB under strictly anaerobic conditions. PHB is naturally available from the lysis of other PHB accumulating bacteria (DAWES AND SENIOR, 1973; UFFEN ET AL. 1971; EMERUWA AND HAWIRKO, 1973).

Fast and sufficiently reproducible data upon the degradation of the polyester were obtained using a pH-controlled bioreactor. The effect of the significantly enhanced weight loss per time is caused by the optimization of the growth conditions of the sensitive test strain and the increase in available polymer surface area by using powder instead of films. The decisive factor influencing and/or limiting the PHB degradation with strain 5a is, however, primarily the pH-value of the degradation medium. PHB degradation ceased when the pH decreased far below 6.8. Increasing the surface area does not seem to have a measurable effect, probably due to enzyme limitation.

The experimental results indicate the involvement of an extracellular enzyme since cell free supernatants and washed PHB pellets from cultures grown on PHB were able to degrade PHB. Yet, microscopic examinations revealed that the cells themselves were not attached to PHB particles. The extracellular PHB depolymerase was produced only after growth with PHB or 3-hydroxybutyrate and to a limited extent on lactate and pyruvate; no activity was detected in cultures grown with crotonate which is a good growth substrate.

The enzyme activity is oxygen sensitive and negatively affected by freezing and thawing. The enzyme exhibits a high adsorption tendency to hydrophobic surfaces and is mainly attached to the PHB surface during the degradation. In this respect the enzyme behaves similar to that of *Pseudomonas lemoignei* (LUSTY AND DOUDOROFF, 1966). It also strongly adsorbs to hydrophobic surfaces such as storage devices and membranes. This strong binding capacity is probably responsible for the low protein recovery (about 68 %) during the purification of the enzyme.

Native PAGE revealed that the purified and concentrated enzyme sample resulted in two distinct protein bands of over 440 and 700 kDa, respectively. The enzyme complexes are most probably polymers which dissociates on SDS-PAGE into its several sub-units. Both protein complexes showed enzymatic activity towards PHB on a native activity gel. This finding together with the existence of two different temperature optima (37 and 55 °C) as well as two pH optima of 7 and 10 makes the involvement of two distinct enzyme complexes depolymerizing PHB probable.

A more detailed discussion will be given in the next chapter.

5. Conclusive Discussion

5.1. Are polyesters principally biodegradable in different anaerobic environments?

Using successively improved test methods, first the anaerobic degradation was evaluated for the different polyesters. Independent of the test set up used, such as weight loss in sludges, biogasification with mixed undefined populations (or even weight loss with isolated strains), the type of sludge or its concentration, a general trend of biodegradability was noticed for the polyesters under investigation:

PHB, PHBV > PCL > SP 3/6, SP 4/6, BTA 40:60.

This implies the impact of polyester properties rather than the nature of the sludge or sediment on anaerobic biodegradation.

Only the natural polyesters PHB and PHBV were degraded to significant amounts in the time scale of a few weeks, with PHB comprising a higher degradation rate than PHBV. This finding is rather surprising, since under aerobic conditions in most cases PHBV exhibited a higher degradation rate than the homopolyester PHB. This fact is usually attributed to the lower cristallinity of the copolyester PHBV (DOI ET AL., 1990; AUGUSTA ET AL., 1993; MERGAERT ET AL., 1993; MERGAERT ET AL., 1996A). In all tests, with microbial consortia (as well as with a pure strain), PHB was degraded faster than PHBV. Probably, the depolymerization products of PHBV, especially 3-hydroxyvaleric acid, and their fermentation products pose a problem to the growth of the anaerobic microorganisms. In agreement with this postulation, preliminary growth substrate investigations and enzyme induction studies performed with a PHB degrading *Clostridium* sp nov. (strain 5a) showed, that 3-hydroxyvaleric acid is a poor growth substrate and does not induce the PHB depolymerizing enzyme. In addition, the biogasification experiments showed that PHBV is faster depolymerized than mineralized. Similarly, REISCHWITZ AND COWORKERS. (1998) detected the accumulation of the intermediate hydrolysis products acetate, propionate, n-butyrate, iso-butyrate and n-valerate during PHBV mineralization using anaerobic sludge cultures as well as a selective sludge culture inoculum. They postulated an inhibition of acetogenic and methanogenic bacteria by the formed organic acids due to an imbalance between the high substrate content to the low bacterial mass, especially with the latter test system.

In comparison to the natural materials (PHB and PHBV), the synthetic polymer PCL showed slower degradation rates. PCL is, however, also definitely attacked by anaerobic microbes.

Here some uncertainties still existed in the literature; some authors could not prove anaerobic degradation for this material (PÜCHNER, 1995; FINK AND SCHÄFER, 1996).

The very low degradation rates of the other synthetic polyesters (SP 3/6, SP 4/6 and BTA 40:60) were surprising, since the same materials are reported to be aerobically easily biodegradable (WITT ET AL. 1997; MARTEN, 2000). However, an enhanced biodegradability of the synthetic polyesters under thermophilic conditions was detected. This finding is congruent with the aerobic situation, where an increase in biodegradation rates for synthetic polyesters was detected under elevated temperatures using unspecific lipases (Marten, 2000). The involvement of different organisms and - as interpreted by MARTEN (2000) - an increase of polyester flexibility with increasing incubation temperature may be one explanation. Secondly, an increase of temperature, generally, has a significant influence on the bioavailability and solubility of organic compounds by a decrease in viscosity and an increase in diffusion coefficient of organic compounds (MÜLLER ET AL., 1998). Consequently, the involvement of lipases or lipase like enzymes in the anaerobic degradation process of synthetic polyesters is possible.

As for BTA, neither elevated incubation temperatures nor blending of BTA 40:60 with starch, a readily biodegradable substrate for many anaerobes, did result in the expected increase in the anaerobic biodegradability. Hence, this aliphatic-aromatic copolyester is anaerobically not biodegradable, at least not under conditions and time scales as those used throughout this work.

From the substrate specificity of the anaerobic microbial isolates observed in this work, it can be suspected that similar enzymatic mechanisms are responsible for polyester degradation under both anaerobic and aerobic conditions. Several lipases from aerobic microorganisms showed unspecific activity against synthetic polyesters (WALTER ET AL., 1995; GAN ET AL., 1997; KLEEBERG ET AL., 1998). The existence of anaerobic lipase producing organisms is to be expected, since lipids are ubiquitous in nature, including anaerobic environments. Yet only few anaerobic lipolytic bacteria have been isolated (mostly from rumen) and characterized being *Anaerovibrio lipolytica* and *Butyrivibrio* strain S2 (MACKIE ET AL., 1991). In addition to these ruminal species several types of *Clostridium botulinum* exhibit lipase activities (HOLDEMAN AND MOORE, 1978). Since some to few anaerobic lipase producing anaerobic bacteria do exist, the anaerobic biodegradability of polyesters such as SP 3/6 should be principally possible.

The low anaerobic biodegradation rates for the synthetic polyesters like SP 3/6, might thus be explained by several enzymatic considerations. First, many of the species involved in anaerobic degradation exhibit slow growth rates (clostridial glucose fermentation might yield only about 3.4 – 3.8 mol ATP per mol glucose (JUNGERMANN ET AL., 1973) as compared to 25 - 38 mol ATP conserved by aerobic organisms). In addition, they are highly sensitive to fluctuations in their physiological environment and are most often highly specialized (GUJER AND ZEHNDER; 1983, ZINDER, 1984). This would mean that although anaerobic lipases do exist, they may be more specific, i.e. are restricted to hydrolyze a special lipid or class of lipids. Regarding PHB depolymerization with the isolate strain 5a for comparison, a comparable enzyme specificity was noticed. The organism showed lower depolymerization rates to PHBV than PHB although they differ only slightly. As anaerobic organisms gain less energy through catabolism and grow slower (as mentioned above) the degradation process might also be slower. Secondly, the enzyme regulation must be considered. It can be postulated that the low biodegradability observed during this work is based on the induction of the lipases by the insoluble and synthetic substrate rather than by the existence of lipase producing anaerobes. In other words, although anaerobic lipases do exist which principally should show activity against SP 3/6, the polyester fails to induce the microbial enzyme system. The higher anaerobic biodegradation rates for PCL observed further support this consideration. The depolymerization products of PCL resemble naturally occurring intermediates of a polymer existing in nature, namely cutin, (the building polymer of plant cuticle), which was clearly proven by MURPHY AND COWORKERS (1996) studying aerobic PCL degradation. Therefore, although being a synthetic substrate, the anaerobic enzyme system (postulating it being a cutinase) is successfully induced leading to the anaerobic biodegradability of this polyester.

Answering the above question, it can be stated that the natural polyhydroxyalkanoates (PHB and PHBV) as well as the synthetic polyester PCL are definitely biodegradable in anaerobic environments. On principle, also the synthetic polyesters SP 3/6 and SP 4/6 are anaerobically biodegradable (even though with lower biodegradation rates), since anaerobic bacteria capable of depolymerizing them were isolated. It has to be stressed at this point, that the commercially relevant polyester BTA 40:60, which is definitely biodegradable under aerobic conditions, can be considered as more or less resistant to anaerobic microbial attack. Thus the question arises if specific hydrolases such as the one isolated from the thermophilic actinomycete *Thermomonospora fusca* (KLEEBERG, 1999), and which differs in its substrate specificity clearly from lipases, play a key role. Basically, information on the

aerobic biodegradation of polyesters can hence be implicated on the anaerobic breakdown (e.g. PHB, PCL). There exist however, differences due to:

1. More restricted catabolic efficiencies (e.g. metabolization of depolymerization intermediates of PHBV)
2. Longer adaptation periods (e.g. the involved induction mechanisms for the non-specific lipase must adapt to SP 3/6)
3. Highly specialized or rare metabolic pathways (e.g. BTA 40:60).

5.2. Which organisms are responsible for anaerobic polyester degradation and what are their characteristics?

Anaerobic digestion is recognized as a complex process involving the coordinated activity of a number of different bacterial trophic groups (McInerny et al., 1980; Gujer and Zehnder, 1983, Zinder, 1984). It was therefore questioned, if single strained anaerobes can exist with polyesters as the sole carbon source. Further more, it was intended to isolate, identify and characterize anaerobes with a high polyester depolymerization potential to be used for basic investigations concerning the anaerobic degradation of polyesters.

A total of 55 anaerobic bacterial strains capable of depolymerizing at least one of the polyesters were isolated from different anaerobic habitats. Four out of five identified polyester depolymerizing isolates belong to the genus *Clostridium*. The clostridia are an extremely diverse group of Gram-positive (Gram-variable species also found), obligately anaerobic or microaerophilic, endospore-forming bacteria, that do not carry out dissimilatory sulfate reduction (Cato and Stackebrandt, 1989). These criteria are met by an otherwise diverse assembly of microorganisms, and the genus *Clostridium* has grown to be one of the largest genera among prokaryotes. Most of them are chemoheterotrophs and obtain energy and biosynthetic precursors by degradation of more or less complex organic matter. Polymers such as starch, cellulose, hemicellulose, pectins, proteins are degraded by depolymerases to produce assimilatable compounds of low molecular weight. Furthermore, clostridia, being spore forming anaerobes, are wide spread since they resist adverse physical conditions. For most clostridia, as for those isolated and characterized in the present work, growth is optimal at pH 6.5 - 7.0 and temperatures between 30 and 37 °C (Andreesen et al., 1989). Clostridia, as anaerobes, evolved early on earth under energy-limited conditions, which may have placed strong selection pressure on the evolution of very efficient catabolic enzymes (Saha et al., 1989).

The bacterial strains isolated throughout this work (a total of 55 isolates) were divided into three separate groups depending on their substrate (polyester) specificities.

5.2.1. Strains degrading selectively natural PHAs (30 strains)

The PHB and PHBV degrading isolates are specialized to depolymerize only the natural hydroxyalkanoates and cannot attack synthetic polyesters and vice versa. Two strains were taxonomically and physiologically identified as *Clostridium* sp. nov. and showed several similarities to *C. homopropionicum*. It was proven during the present study that the latter organism can also depolymerize PHB. In addition, the strain *Ilyobacter delafieldii* previously reported to degrade PHB (JANSSEN AND HARTFOOT, 1990; JANSSEN AND SCHINK, 1993) obviously must be re-classified as *Clostridium delafieldii* according to the new taxonomic data obtained during this work. Another *Clostridium* sp. was stated by MERGAERT ET AL. (1996B) to depolymerize natural PHAs. These findings suggest that anaerobic PHB depolymerizing bacteria are mainly found among clostridia.

Both new isolates are metabolically highly restricted but metabolize the depolymerization product (monomer 3-hydroxybutyrate). The organisms seem to be specialized on PHB degradation and metabolization. The involved enzyme is most probably a specific PHB depolymerase, a class of hydrolases which intensively has been investigated for aerobic microorganisms.

Carbon catabolite repression by glucose was observed for the organisms degrading hydroxyalkanoates and hence problems occurred with preservation of the organisms and/or instability of degradation character. For many isolates the PHB degradation ability was lost in absence of nutritional stress due to the presence of readily metabolizable substrates. It is known for clostridia that easily degradable substrates might mask abilities for biosynthesis and biodegradation and hence extracellular enzymes of polymeric substrates (ANDREESEN ET AL., 1989; MITCHELL, 1998). It is assumed, that the presence of glucose as a growth substrate results in what is known as inducer (3-hydroxybutyrate) expulsion as has been observed for other clostridia (DIEZ-GONZALEZ AND RUSSELLL, 1996; BEHRENS ET AL., 1997; MITCHELL, 1998). Thus the PHB depolymersing enzyme is not induced and PHB is not degraded.

The instability found by the isolated strains may point to the involvement of a plasmid encoded character rather then a chromosomal one. Indeed, plasmids have been found in many clostridia (YOUNG ET AL., 1989). Similarly, WIEGAND AND COWORKERS (1999) observed

the loss of the degradation ability by the isolated aerobic BAK 1095 (a biodegradable random copolymer of polyester amide developed by Bayer AG) when there was temporarily no selection pressure by the growth on BAK 1095 as the sole source of carbon. They explained this findings by an extrachromosomal codation of the degradation character.

5.2.2. Strains degrading selectively PCL (16 strains)

PCL degrading strains are also specialists since they only showed depolymerization activity towards PCL. No organism originally screened on PCL, degrades the other synthetic polyesters SP 3/6, SP 4/6, BTA 40:60 or the natural PHAs.

Two strains - identified as *Clostridium* sp. nov. - are lipase negative, although most of the described aerobic PCL depolymerizing enzymes are reported to be lipases (TOKIWA ET AL., 1988; ODA ET AL., 1997; MURPHY ET AL., 1998). The strains did not metabolize the depolymerization products, i.e. the monomers of PCL. The involved enzyme system must hence be a hydrolyzing enzyme, which depolymerizes PCL probably due structural similarities between its depolymerization products and those of another structurally similar polymer, such as for example cutin. Similarly, literature on aerobic PCL-degradation also gives evidence about the involvement of cutinases in PCL depolymerization (NISHIDA AND TOKIWA, 1994B; MURPHY ET AL., 1996). Cutinases are enzymes degrading cutin, the polyester structural component of plant cuticle and are secreted by many phytopathogenic microorganisms (BAKER AND BATEMANN, 1978; FETT ET AL., 1992). Also MURPHY AND COWORKERS (1996) presented genetic, regulatory and enzymatic evidence for the involvement of a cutinase in aerobic PCL degradation. In addition, they showed that PCL dimers and trimers are structurally similar to natural inducers of cutinase. It is therefore possible that anaerobic PCL degradation follows the same principle as aerobic PCL degradation.

5.2.3. Strains degrading synthetic polyesters (9 strains)

From the 9 strains able to degrade also other synthetic polyesters than PCL (none of them did attack the natural polyhydroxyalkanoates) only one isolate - taxonomically characterized as *Propionispora* sp. nov. - showed a wide substrate spectrum within the synthetic polyesters. (Since this genus (*Propionispora vibroides* nov. gen., nov sp.) has been recently established by BIEBL ET AL. (2000) not much information is available about these organisms). It is however clear, that the isolated strain did not metabolize the depolymerization products of the polyesters. The involved depolymerizing enzyme seems to be an unspecific degrading enzyme, probably an unspecific lipase. This behavior is not rare in nature as most often,

microbial activity against synthetic organic materials is a result of broad enzyme specificities (AMINABHAVI ET AL., 1990). Likely, several strains of clostridia such as *C. thermocellum* synthesize xylanase enzymes, for example, but grow poorly if at all, on xylan owing to an inability to accumulate the degradation products (GARCIA-MARTINEZ ET AL., 1980; MORAG ET AL., 1990; BRONNENMEIER AND STAUDENBAUER, 1993; HAZLEWOOD AND GILBERT, 1993).

It is worth stating that the polyesters were depolymerized with this particular strain in the order of their ascending melting points also pointing to the involvement of a lipase or lipase like enzyme:

SP 3/6 (40 °C) > PCL (60 °C) > SP 4/6 (62.3 °C) > BTA 10:90; 20:80 (62.5 °C)

Similar observations were made by MARTEN (2000) investigating the correlation between the structure of different synthetic polyesters and their enzymatic hydrolysis, and clearly showed that lipases generally degrade synthetic polyesters non-specifically. Marten found that the degradability of different polyesters with a given lipase increases with the decrease of the melting temperature of the polyester. Decisive for this phenomenon is not a selectivity of the active center of the lipase but merely the difference between the melting temperature of the given polyester and the incubation temperature. The lower this temperature difference is, the higher the observed hydrolysis of the polyester material. Consequently, the generally low specificity of the involved anaerobic degradation enzyme to the different structural characteristics in the polymer chain is comparable to the aerobic situation. The main reason for the differences in the biodegradability observed for the different polyesters – similarly to the aerobic biodegradabilities - is due to a different behavior caused by specific parameters of the polymer matrix of each specific polyester.

Obviously, at least three different enzyme systems are involved in the anaerobic degradation of the different polyesters. Thus, this observation is congruent to the aerobic situation where also three different kinds of enzymes: PHB depolymerases, lipases and cutinases are discussed to be involved in polyester degradation. Non of the aerobic PHA depolymerases shows significant lipase activity or attacks synthetic polyesters due to an inability to bind a long-chain triacylglycerol or hydrolyze the lipase substrate (JAEGER ET AL., 1995). However, several lipases hydrolyze polyesters of ω-hydroxyalkanoic acids such as PCL and BIONOLLE. Cutinases on the other hand, are serine hydrolases for primary alcohol esters (KAZLAUSKAS, 1994; SVENDSEN, 1994) which depolymerize cutin specifically, and cutin like polyesters as PCL non-specifically. Concerning at least the enzymatic respect of the anaerobic polyester degradation, therefore, strong parallels to the anaerobic one seem to exist.

5.3. Polyesters in anaerobic waste management systems

Although anaerobic biodegradation of polyesters basically seems to follow the same strategies as the aerobic biodegradation, the introduction of polyesters, and probably most of the biodegradable plastics, in anaerobic waste treatment processes must be critically questioned. In light of the data obtained during the present work, it seems that the anaerobic degradation of synthetic polyesters (with the exception of PCL) is very slow. The successful enrichment of anaerobic single strains depolymerizing these polyesters (BTA 40:60 depolymerizing anaerobes were not isolated) required about 18 months in this work. Thus, even though anaerobic microorganisms might exist which are equipped with the required (non-specific and broad spectrum) hydrolyzing enzyme(s), it remains questionable if such organisms can establish themselves, in order to predominate in the consortia as effective degraders. Considering that most anaerobic waste treatment processes have a residence time for the organic matter of about two to three weeks, and predominantly are run under mesophilic conditions, a complete biodegradation of synthetic polyesters (at least for those examined) is not to be expected.

However, in practice a final aerobic treatment step (composting for the purpose of stabilization) is usually part of the technical procedures and here the polyesters may finally be degraded. Given that the items (e.g. films) made of biodegradable polyesters do not disturb the aerobic process (which is not to be expected if they were at least disintegrated in the anaerobic step), a final degradation in the last aerobic section of the process would occur.

5.4. Investigations on PHB degradation with a selected strain

Interpreting the obtained degradation data in light of carbon catobolite repression, it becomes obvious that heterogeneous degradation results may be obtained for the same polyester in different sludges (containing various and varying amounts of readily metabolizable carbon sources) even though they inhabit the same or similar organisms. Therefore, results obtained with unidentified mixed microbial populations can only be interpreted, if the factors influencing the responsible degrading organisms are known and understood.

In addition, it became clear from the medium optimization experiments that the degradation potential of the isolated organisms is greatly influenced through variation of the growth medium. Comparably, the different sludges and sediment used in the comparative

degradation studies vary in their composition and may or may not satisfy the different nutritional requirements of the degrading organisms, thereby limiting their degradation capabilities. Thus the development of a defined and optimized test for polyester degradation, using defined microorganisms (individual strains) was necessary.

5.4.1. Improved test system for PHB degradation with strain 5a

Using PHB powder instead of pressed films in degradation tests under pH-controlled conditions, the isolated PHB degrading strain, *Clostridium* sp. nov. strain 5a, was successfully used in improved and significantly accelerated degradation tests yielding sufficiently reproducible data upon the degradation of the polyester. Instead of a time scale of months, results about the anaerobic degradation of PHB could be obtained within 3 days in a pH-controlled bioreactor system. Only the use of a defined mineral medium without any other relevant carbon sources makes it possible to use the polymer powder. In sludges the recovery of non degraded polymer powder would be very problematic.

The effect of the significantly enhanced weight loss per time is caused by both, the optimization of the growth conditions of the sensitive test strain by pH-control and the increase in available polymer surface area by using powder instead of films. The non-linear impact of the available surface area might be the relatively low cell growth resulting in a low enzyme concentration, i.e. the enzyme concentration rather than the available surface area is the limiting factor. However, considering the anaerobic breakdown of PHB as it occurs in nature (e.g. after the lysis and death of other bacteria containing PHB) the occurrence of high PHB concentrations is not to be expected. Consequently, a higher PHB depolymerase activity or concentration might not be necessary.

5.4.2. PHB degrading, anaerobic enzyme system from strain 5a

The limited metabolic capacity of this strain and its ability to grow on PHB suggest that the organism may be specialized to degrade exogenous PHB under strictly anaerobic conditions upon the death and lysis of other bacterial cells. Several anaerobic bacteria are known to accumulating PHB as a carbon/energy reserve material (DAWES AND SENIOR, 1973). Examples for such bacteria are *Rhodospirillum rubrum* (UFFEN ET AL. 1971), *Clostridium botulinum* (EMERUWA AND HAWIRKO, 1973), *Syntrophomonas wolfei* (MCINERNEY, ET AL., 1981) and a number of sulfate reducing bacteria (WIDDEL, 1980), etc.

Experimental results indicate that the initial PHB breakdown is catalyzed by an extracellular PHB depolymerase which is produced only after growth with PHB or 3-hydroxybutyrate and

to a limited extent with lactate and pyruvate. Similar to the isolated PHB depolymerase, starch hydrolysis by saccarolytic clostridia via amylolytic enzymes is induced by starch and its degradation products (ANTRANIKIAN, 1990).

As mentioned before, the anaerobic PHB depolymerase synthesis is depressed in the presence of fermentable growth substrates other than PHB or the monomer. Evidence exists that – similar to the isolated enzyme - synthesis and secretion of aerobic PHB depolymerase are subject to a double regulatory control: by catabolite repression in the presence of a more readily utilizable C-source and by derepressing elicited in the absence of a utilizable substrate and (JENDROSSEK ET AL., 1993B). Also, cellulase synthesis responds to the energy state of the cell, since activity showed a direct relation ship to the cellular ATP (NOCHUR ET AL., 1993).

It remains questionable how an insoluble and hydrophobic polymer such as PHB which cannot enter the cell, is able to induce PHB depolymerizing enzyme activity. After LIN AND KOLATTUKUDY (1978) microorganisms secret continuously low amounts of various extracellular hydrolyzing enzymes into their surrounding. The depolymerization products thereby produced are taken up into the cell, where they can induce the synthesis of appropriate amounts of the required or favorable hydrolyzing enzyme. The induction of the anaerobic PHB depolymerase in the present work by both the polymer and the monomer supports a comparable induction mechanism.

The isolated anaerobic PHB depolymerase was preliminary characterized. The purified and concentrated enzyme sample, resulted in two distinct high molecular weight protein bands of over 440 and 700 kDa, while aerobic PHA depolymerases usually have relatively small M_r of below 100 kDa (JENDROSSEK, 1998). Both isolated protein complexes showed enzymatic activity towards PHB. The existence of two PHB-active protein complexes, with two different temperature optima (37 and 55 °C) as well as two pH optima of 7 and 10 makes the involvement of two distinct enzyme complexes depolymerizing PHB probable. The existence of two different PHB depolymerases for one microorganism seems on the first view unusual and many aerobic PHA-degrading bacteria apparently excrete only one depolymerase. However, for *P. lemoignei* six depolymerases were identified (BRIESE ET AL., 1994; JENDROSSEK, 1998) which differ slightly in their biochemical properties. It is therefore likely, that other bacteria also have more than one depolymerase.

It has to be stressed, that this is the first time that an anaerobic PHB depolymerizing enzyme has been characterized.

5.4.3. Comparison of anaerobic PHB depolymerization with cellulose decomposition by clostridia

There is no information available in literature on anaerobic PHB depolymerases; anaerobic depolymerases of other polymeric substrates can however be considered for comparison. Anaerobic cellulose degradation seems to be a good example due to several parallels. Cellulose – a crystalline polymer - is composed of linear chains of β-1,4-linked D-glucose residues. The anaerobic depolymerases isolated in the present work are protein complexes of unusually high molecular weights (of over 440 and 700 kDa) if compared to aerobic PHB depolymerases. However, the anaerobic cellulase activity in *Clostridium thermocellum* (NG AND ZEIKUS, 1981; JOHNSON ET AL.; 1982, KOHRING ET AL.; 1990; KRUUS ET AL., 1995) and other clostridial species (LAMED ET AL., 1987; DOI ET AL., 1994A) is also found predominantly in a large, extracellular multi-protein complex which has been called the cellulosome (LAMED ET AL., 1983; FELIX AND LJUNGDAHL, 1993). The cellusome apparently consists of around 20 polypeptides with a very high total molecular weight of over 2000 kDa. In contrast to the anaerobic PHB depolymerase, the enzyme complex remains associated with the cell surface (fig.1) and mediates binding of the cells to cellulose (ANDREESEN ET AL., 1989), but may be released from cell surface late in growth.

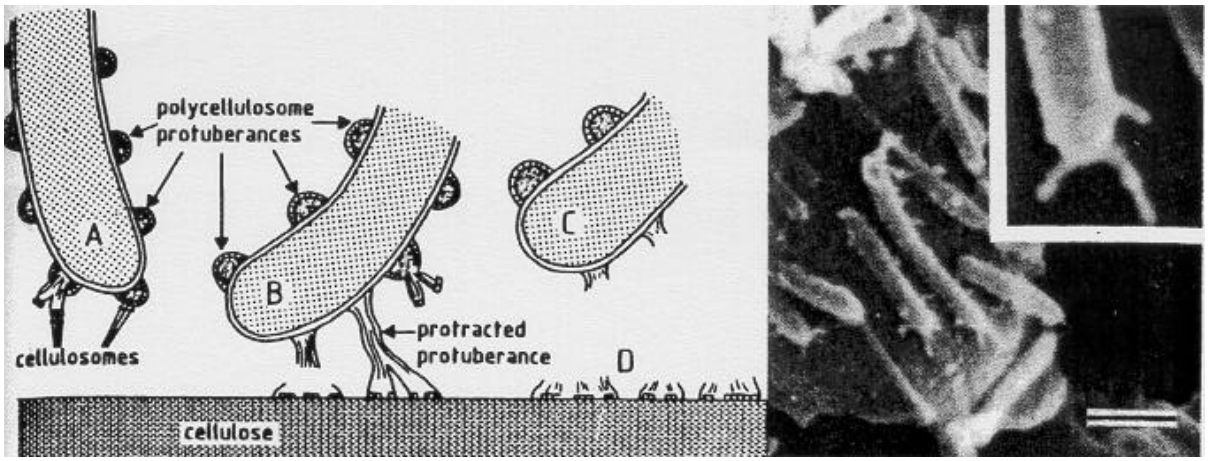


Fig. 1. Interaction of *C. thermocellum* cells with cellulose mediated by protracted polycellulosomal protuberances. Left: cell A, prior to contact; cell B, following contact; cell C, following attachment. Right: SEM of cationized ferritin stained cells of *C. thermocellum* attached to cellulose. Bar, 1.0 µm. (LAMED AND BAYER, 1987.)

Similar to the affinity of the cellulases from clostridia to the substrate cellulose, the PHB depolymerase activity in the present work was mainly associated with the PHB granules. Once all the PHB granules were degraded, enzyme activity was detectable in the culture

supernatant. This strong binding of the PHB-depolymerase isolated in this work to hydrophobic surfaces resulted in a relative low protein recovery (about 68 %) during the purification of the enzyme. With respect to the strong binding affinity, the anaerobic enzyme also behaves similar to the aerobic PHB depolymerase of *Pseudomonas lemoignei* (LUSTY AND DOUDOROFF, 1966) which also strongly adsorbs to hydrophobic surfaces such as storage devices and membranes.

Experimental findings suggest the ability of separated subunits of the anaerobic PHB depolymerase enzyme complexes to reassemble. The filtrate – passed through a membrane with a cut off of 100 kDa - showed clear protein bands with molecular weights over 440 kDa as well as low enzyme activity. Similarly, HAZLEWOOD ET AL. (1990) found, that the cellulase enzyme can exist both in full-length as well as in truncated forms. Also MATANO AND CO-WORKERS (1994) proved that individual components of the cellulose degrading enzyme can reassemble to form the active complex in presence of the substrate.

Basically it can be stated, that anaerobic PHB degradation follows the same strategy as aerobic degradation does with additional parallels to other anaerobic depolymerizing enzymes. However, several questions are still open. It is still not clear, whether dimers and trimers appear as primary depolymerization products of the enzyme isolated from this particular strain. Yet, RESCHWITZ ET AL. (1998) proved the accumulation of four different dimeric esters of 3-hydroxybutyrate and 3-hydroxyvalerate during the degradation of PHBV in an anaerobic sludge. Thus, at least the production of dimers of 3-hydroxybutyrate by strain 5a must be postulated. Similarly, for aerobic PHB depolymerases, monomers (*Comamonas* sp.) or oligomers, i.e. mono- to trimers, (*Pseudomonas* sp.) depending on the depolymerase have been detected (KASUYA ET AL., 1999). Further work on screening and identification of possible occurring oligomers is therefore necessary. In addition the substrate specificities of the purified enzyme still have to be investigated. It would also be interesting to compare the two isolated PHB depolymerizing protein complexes with respect to structural similarities or differences. Most important, however, is the analysis of the amino acid sequence to definitely classify these enzymes among other aerobic PHB depolymerases. Finally, the investigation of regulation mechanisms governing enzyme induction and repression definitely represents an interesting field of research.

As evidenced by the present study, anaerobic polyester degradation is a more wide spread phenomenon than was previously thought. Very interestingly, characteristic patterns in terms of substrate range were found in the present work. Care must however be taken, to avoid

over-interpretation because of the limitations of sampling sites. However, these findings are very important for understanding the organisms responsible for the anaerobic biodegradation of biodegradable plastics in situ. Further studies with the obtained new isolates should provide interesting insights into potentially novel mechanisms for anaerobic polyester degradation. No doubt, the enormous use of plastics will be the driving force for further research on their anaerobic biodegradation processes.

6. Summary

Systematic studies on the anaerobic biodegradation of the natural polymers poly(hydroxybutyrate) (PHB), poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), of some industrially important biodegradable synthetic polyesters such as poly(e-caprolactone) and aliphatic-aromatic copolyesters from 1,4-butanediol, terephthalic acid and adipic acid (BTA-copolyesters) and corresponding pure aliphatic polyesters based on 1,4-butanediol and adipic acid (SP4/6) or 1,3-propanediol and adipic acid (SP3/6), respectively were performed. First, the degradation behaviour was examined in different anaerobic environments (anaerobic waste water sludges, sludge from a biowaste treatment plant and anaerobic river sediment) using laboratory simulation tests (weight loss determination) and measurement of mineralization via biogas evolution). From the enriched populations of the simulation experiments individual anaerobic strains able to depolymerize the different polyesters were isolated and selected strains were identified. With these strains defined and accelerated laboratory degradation tests were established. Finally, an extracellular PHB degrading anaerobic enzyme was identified.

In contrast to most reported studies under aerobic conditions the copolyester PHBV degraded anaerobically slower than PHB, in the sludge simulation tests and the defined laboratory tests, as well. Thirty PHB degrading organisms could be isolated from the different sludges using a specially developed method based on clear zone formation on agar plates. Two of the isolates were identified as *Clostridium* sp. nov.. PHB degradation was sensitive to different carbon-source supplements added to the synthetic media. In addition, the ability of the strains to degrade PHB was maintained only by applying suitable cultivation conditions.

Beside the natural polyesters, all synthetic aliphatic polyesters were anaerobically degraded, too, while the aliphatic-aromatic copolyesters turned out be resistant to biodegradation above a certain content of terephthalic acid. A number of anaerobic strains (total of 29) were isolated which degrade PCL and the other aliphatic polyesters (to our knowledge the first time for synthetic polyesters). Two of the isolates were identified as *Clostridium* sp. nov. and *Propionispora* sp. nov. None of these 29 isolates degraded the natural polyhydroxyalkanoates PHB and PHBV and none of the PHB degrading organisms was able to attack the synthetic aliphatic polyesters under investigation. This can be regarded as a indication of the presence of generally three different enzyme systems as observed for aerobic degradation, too. Aerobically polyhydroxyalkanoates are depolymerized by very

specific PHB-depolymerases, while synthetic polyesters have shown to be preferably attacked by unspecific lipases. Some of the isolates depolymerizing only PCL may secrete lipase-like enzymes which depolymerized only PCL and hence are possibly cutinases.

With one PHB-degrading strain an improved and accelerated laboratory test system was developed to be used for studies of the anaerobic degradation mechanism of PHB. By optimizing and controlling the environmental conditions and by applying PHB powder instead of films it was possible to run degradation tests in defined synthetic environments in a time scale of hours instead of months required under natural conditions. It turned out that the PHB degradation process was mainly influenced by controlling the physiological conditions by especially the pH value; the increase in the available surface area (PHB-powder instead of films) was subordinate.

Extracellular PHB-depolymerizing enzymatic activity could be identified with help of a specially developed test system. Main characteristics of the purified enzyme and its regulation were investigated and compared with aerobic PHB depolymerases and other anaerobic degrading enzymes.

7. Materials and Methods

7.1. Polymers

The chemical structure of the different linear polyesters chosen for the degradation studies are summarized in Fig 7.1.

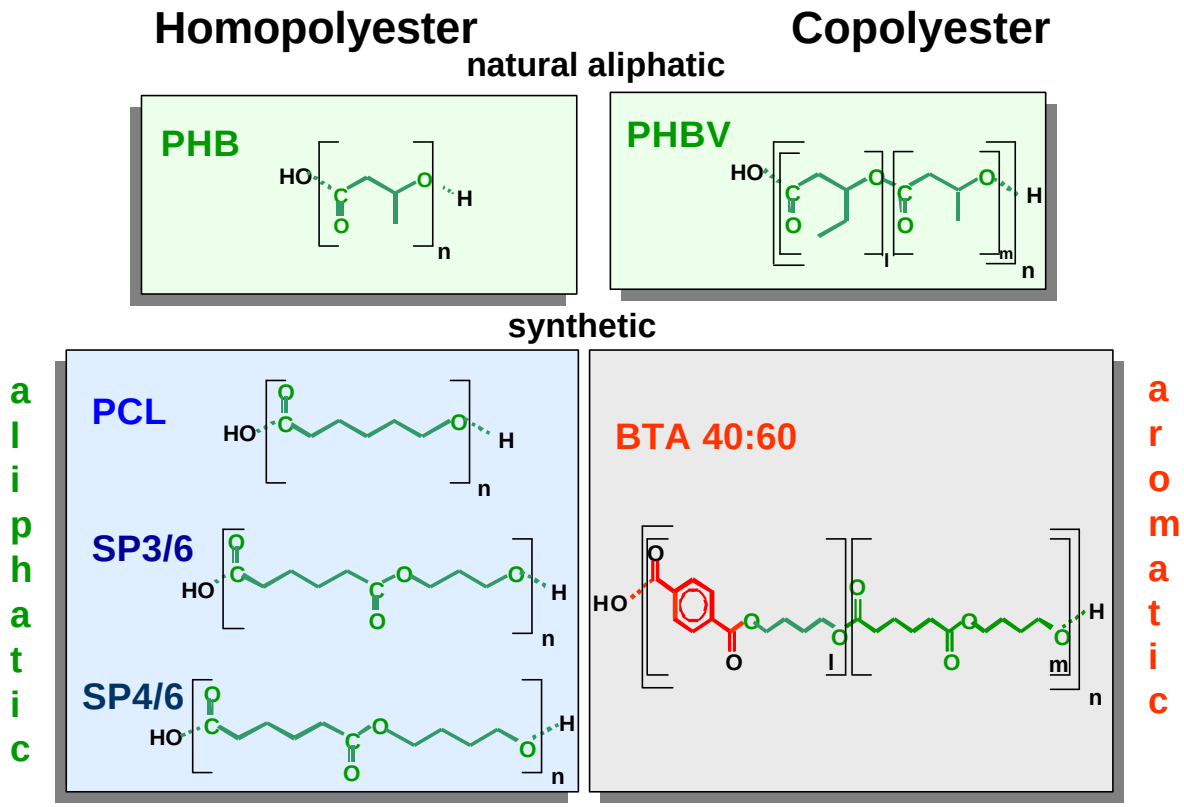


Fig. 7.1. Chemical structure and classification of the linear polyesters used during this study.

Table 7.1 summarizes the chemical components and composition and physical characters as melting temperatures and molar masses, as well as the sources of the polyesters.

Table 7.1: **Chemical structure, composition, melting temperatures and average molar masses of tested polymers.**

Polymer	Component(s)	T_m [a] (°C)	M_w [b] (g/mol)	Source
PHB	3-hydroxybutyrate	180	540000	ICI, Billingham, United Kingdom (as Biopol BX G08)
PHBV	3-hydroxybutyrate / 3-hydroxyvalerate (10 mol%)	180	397000	ICI, Billingham, United Kingdom (as Biopol BX P027)
PCL	ε-caprolactone	60	50000	Polysciences, Inc. Warrington, USA
PCL 787	ε-caprolactone	63	200000	Novamont S.p.A., Novara, Italy.
PCL-S MATER Bi ZF03U/A	ε-caprolactone and starch (40 %)	63	187000	Novamont S.p.A., Novara, Italy.
SP 3/6	1,3-propanediol / adipic acid	44,1	38000	GBF, Braunschweig
SP 4/6	1,4-butanediol / adipic acid	62,3	40000	GBF, Braunschweig
BTA 40:60	1,4-butanediol / adipic acid (60 mol%) / terephthalic acid (40 mol%)	99	47600	Hüls AG, Marl
BTA ZK1094	1,4-butanediol / adipic acid (45 mol%) / terephthalic acid (55 mol%)	120	66500	Novamont S.p.A., Novara, Italy.
BTA-S MaterBi NF01U	1,4-butanediol / adipic acid (45 mol%) / terephthalic acid (55 mol%) and starch (32 %)	92	145000	Novamont S.p.A., Novara, Italy.

a) T_m: melting temperature; maximum of DSC melting peak.
b) M_w: weight average molar mass (determined by gel permeation chromatography, based on polystyrene calibration).

7.1.1. Polyester sample preparation for degradation tests

In order to work under defined and reproducible conditions, the polyesters were processed into thin films by compression molding using a hydraulic press (Ident.-Nr. 062566, Perkin Elmer, Überlingen) with two thermostatable metal plates (PIN 15515, Specac, England) as described by WITT ET AL. (1995). The polymer powder was placed between two teflon coated paper sheets separated by an metal spacer (varying thicknesses were used to determine the film thickness required for each specific test). The polyester material (powder, granules, thick pre-pressed films) was then compression molded for 2 minutes applying pressure through a weight of 2 tones. The temperature was chosen 5 °C below the melting temperature (see table 7.1). SP 3/6 was compression molded without applying pressure. PCL 787 and PCL-S MaterBi ZF03U/A as well as BTA ZK1094 and BTA MaterBi NF01U were available as sheets (thickness: 74, 30, 55, 40 µm, respectively). Using punches with defined diameters films of defined surface areas were cut. Table 7.2 summarizes the various film sizes and degradation surface areas as well as test volumes and test devices used in the different degradation tests.

Table 7.2. Diameters and surface areas of films used in the different degradation experiments

Degradation test	Chapter	Film Diameter (mm)	Degradation Surface (cm^2)	Test volume / vial volume (ml/ml)
I. Degradation tests with unidentified mixed cultures				
i. Weight loss determination				
LS, WWS, AS	4.1.1., Fig. 4.1.	25	39.3	150 / 250
LS (10 and 100%)	4.1.1., Fig. 4.2.	25	39.3	150 / 250
Mesophilic versus thermophilic	4.1.1.1., Fig 4.3; 4.4	19	22.7	150 / 250
Influence of blending	4.1.1.2., Fig 4.5–4.8	19	22.7	150 / 300
ii. Biogasification				
Predigested sludge	4.1.2.1., Fig. 4.9	19	22.7	150 / 250
Diluted sludge	4.1.2.2., Fig. 4.10	19	22.7	150 / 250
II. Degradation tests with isolated strains				
Medium optimization	4.2.2.2., Fig. 4.16	9	5.1	10 ml, Hungate tube
Strain selection	4.2.3.1., Fig. 4.19	9	5.1	10 ml, Hungate tube
Medium optimization	4.2.3.1.1., Fig. 4.20	9	5.1	10 ml, Hungate tube
Plate test with strain 5a	4.3.1., Fig. 4.27	25	19.6	Agar plates

7.1.2. Sample sterilization

The vacuum dried and pre-weighed films were sterilized by either of the two following methods:

a-UV irradiation (WALLHÄUSER, 1984).

Each film surface of the polyester films was exposed for 15 min to UV-irradiation using an UV lamp (UVC 30; Hereaus, Holding GmbH, Hannover, Germany; 254nm, 6W cm^{-2} with a distance of 20 cm). The films were placed on a irradiation surface of 18 cm x 38 cm at a constant distance of 15 cm from the irradiation source.

b-Hydrogen peroxide treatment (WALLHÄUSER, 1984).

For degradation tests in liquid cultures the processed films were inserted singly in small petri dishes (Ø = 35 mm, Greiner, Frieckenhausen) and exposed per each side to 10 % (vol/vol) H_2O_2 for 1 hour. After removing the H_2O_2-solution the films were dried at room temperature over night and washed thereafter in three subsequent volumes of 500 ml sterile distilled water using a sterile forceps.

c-Autoclaving

Optionally, the processed and pre-weighed PHB and PHBV films, having a melting temperature of 180 °C, were sterilized in glass petri dishes at 121 °C and a pressure of 1 bar for 20 minutes (Matachana, Zirbus, Osterode). If the polyesters were used as powder for agar plate clear zone tests PHB or PHBV powder was sterilized together with the medium constituents. Direct autoclaving of polyester films of the other materials was not possible due to their low melting points and the tendency to agglomerate (see 7.2.3.).

7.2. Microbiological investigations

7.2.1. Source of inocula

Three different sources of technically managed and controlled disposal systems were used as a source of anaerobic bacteria for all degradation and isolation tests:

a) Anaerobic sludge from a waste water treatment plant (**w**aste **w**ater **s**ludge: ***WWS***) from an anaerobic digester of a municipal waste water treatment plant (Gifhorn, Germany).

b) Anaerobic methane producing sludge collected from an anaerobic laboratory reactor of the Institute for Technology of Carbohydrates (Technical University, Braunschweig, Germany) fed with waste water from sugar industry (**l**aboratory **s**ludge: ***LS***)
c) **T**hermophilically treated **b**io**w**aste (***TBW***) from the anaerobic biowaste treatment plant in Watenbüttel, Germany.

The fourth microbial source was a natural environment:
Anaerobic river **s**ediment ***(AS)*** from Spittelwasser, a side arm of the Elbe river, Germany.

The sludges were used in part for preliminary degradation tests and the preparation of polyester degrading enrichment cultures while the other parts were stored under nitrogen at 4 °C.

7.2.2. Media for cultivation and degradation experiments

The compositions of the media used throughout this work are listed in Table 7.3. The redox-indicator resazurin was added to all media at a final concentration of $1x10^{-4}$ to $2x10^{-4}$ %. Media showing the characteristic red to pink color - pointing to the presence of oxygen - were discarded. Media sterilization occurred by autoclaving at 121 °C and 1 bar pressure for 20 minutes (Matachana, Zirbus, Osterode).

The pH values of the complex media were adjusted prior to sterilization with 0.1 M sodium hydroxide or hydrochloric acid to the desired value. The mineral media were readjusted to the desired pH value after the addition of the reducing agents using sterile 0.1 M sodium hydroxide or hydrochloric acid under anaerobic and aseptic conditions either under the anaerobic glove box (Coy laboratory Products inc., Michigan, USA) with sterile pipettes or with sterile N_2 flushed syringes when “Hungate-tubes” were applied.

Methods for the preparation of anaerobic media such as anaerobic gassing of all cultivation devices, the preparation of oxygen free solutions and the anaerobic cultivation were essentially those of HUNGATE (1968).

Table 7.3: **Cultivation media.**

Mediuma	Composition (per 1 l) / Reference
RAAM	Revised Anaerobic Mineral Medium (SHELTON & TIEDJE, 1984)
GV-Medium	DSM medium: DSM 500, DSMZ. 1998. Catalogue of strains
DSM 503	DSMZ. 1998. Catalogue of strains.
DSM 503 with serine	DSMZ. 1998. Catalogue of strains.
Mineral salt media[b] (MSV)	K_2HPO_4, 0.35 g; KH_2PO_4, 0.27 g; NH_4Cl, 0.5 g; $CaCl_2x2H_2O$, 0.075 g; $FeCl_2x4H_2O$, 0.02 g; $MgCl_2x6H_2O$, 0.1 g, trace element solution 1 ml, vitamin solution 1 ml, selenite/tungstate solution, resazurin, cysteine-HCl, Na_2S.
Methane sludge supernatant medium[b]	
LSS-MSV	Mineral salt medium with the addition of 2.5 % methane sludge supernatant (see 2.4).
Additives	
Trace element solution	$MnCl_2x4H_2O$, 0.5 g; H_3BO_3, 0.05g; $ZnCl_2$, 0.05 g; $CuCl_2x2H_2O$, 0.03 g; $CoCl_2x6H_2O$, 0.5 g; $NiCl_2x6H_2O$, 0.05 g; $Na_2MoO_4x2H_2O$, 0.01 g. Demineralized water was added to complete 1 liter.
Selenite/Tungstate solution	NaOH, 0.5 g; $Na_2SeO_3x5H_2O$, 3 mg; $Na_2WO_4x2H_2O$, 4 mg. The salts were added to 1 liter de-mineralized water and stored at 4 °C.
Redox indicator	Resazurin solution: 0.1 % (w v^{-1}) in distilled H_2O.
Vitamin solution	Biotin, 2 mg; folic acid, 2 mg; pyridoxalhydrochloride, 10 mg; thiamindichloride, 5 mg; riboflavin, 5 mg; nicotinic acid, 5 mg; DL-calciumpantothenate, 5 mg; vitamin B 12, 0.1 mg; p-aminobenzoate, 5 mg; lipoic acid, 5 mg; were dissolved in 1 l distilled water. The solution was membrane filtered (pore size 0.2 µm) and stored at 4 °C. 10 ml of the sterile solution were added to 1 liter autoclaved medium.
Reducing agents	Cysteine-hydrochloride solution (0.1 %). Na_2S solution (0.1 %). The filter sterilized solutions were added to the sterile medium to a final concentration 0.025 %.
Complex media[c]	
PYG medium	DSMZ. 1998. Catalogue of strains.
Anaerobic-***TVLS*** medium	Merck, Darmstadt, Germany
Brewer's anaerobic medium ***(Br)***	Merck, Darmstadt, Germany
Thioglycolate medium ***(TG)***	Merck, Darmstadt, Germany

[a] 20 g agar l^{-1} was added to all media; pH was adjusted to 7.2 ± 0.2; media were autoclaved for 15 minutes at 121 °C.

[b] mineral salt and methane sludge supernatant media used for the enrichment of and the screening for test of organisms.

[c] complex media used for isolation and cultivation of isolated strains.

7.2.3. Preparation of clear zone plates

a- Clear zone plates with natural hydroxyalkanoates (PHB and PHBV agar plates)

For clear zone tests (AUGUSTA ET AL., 1993) PHB and PHBV powder was added to MSV medium (Table 7.3) at a final concentration of 0.1 % (w/v) and ultra-sonicated for five to seven minutes at 90 duty cycles using a Branson sonifier (Branson Ultrasonic Cooperation, Danburry, CT, USA).

b- Clear zone plates with synthetic polyesters (PCL, SP3/6, SP4/6, BTA agar plates)

For the synthetic polyesters turbid agar plates were prepared by a newly developed emulsion technique. The polyester (0.25 g) was dissolved in 5 ml methylene chloride and the solution was then emulsified by sonication into 250 ml of the MSV- or MSS-MSV medium containing 1.5 % (w v^{-1}) agar-agar (Difco, Detroit, Michigan, USA). The emulsion was then stirred continuously while heating for at least 30 minutes to evaporate the solvent completely. Once the characteristic color of resazurine had changed from pink to colorless, the dissolved oxygen as well as the solvent had been completely driven out of the medium. No rest solvent was detected in the medium and growth of the organisms was not affected by this method of polyesters plate preparation. After sterilization by autoclaving homogeneously turbid plates were obtained. The pH was adjusted to pH 7 ± 0.2. The autoclaved medium resulted in homogenous opaque plates. Optionally yeast extract, 0.1 % (w v^{-1}), (Serva, New York, USA) was added to the degradation media.

c- Degradation medium optimization

For degradation medium optimization the mineral media listed in Table 7.3 were supplemented with one of the different polyesters under investigation as a sole source of carbon. Alternatively, an additional carbon source was added as a co-metabolite such as yeast extract (Serva, New York, USA), glucose, sodium acetate, sodium tri-citrate (Merck, Darmstadt, Germany) and sodium crotonate (Sigma, St. Louis, USA) at a final concentration of 0.1 % (w v^{-1}).

7.2.4. Preparation of laboratory sludge supernatant

Methane sludge supernatant (***LSS***) (Table 7.3), as a source of growth factors, was prepared by centrifugation of the methane sludge two times at (11000 rpm, 10 °C and 30 min), filtering the supernatant twice through a folded filter (Schleicher and Schüll 595-1/2, diameter: 240 mm) and autoclaving the filtrate twice for 20 minutes at 121 °C before adding it to the MSV medium at a final concentration of 2.5 % (v v^{-1}) or as otherwise stated in the text.

7.3. Roll tube preparation

For screening of polyester degrading anaerobes the roll-tube technique was chosen for its simplicity. "Hungate tubes" (15 ml) containing 3 ml of the suspended polyesters (PHB, PHBV, PCL, SP 3/6, SP 4/6 or BTA 40:60) medium (same medium preparation as in 7.2.3., a and b) were cooled to 47 °C after sterilization and inoculated directly from the enrichment cultures or serial dilutions prepared thereof. The tubes were then immediately cooled by rolling over a cold surface forming a thin film of medium on the tube surface. After incubation for one to two weeks at 35 °C with a nitrogen atmosphere in the head space area, tubes were inspected for clear zone formation.

7.4. Incubation temperature

All plates were incubated anaerobically in an anaerobic glove box (Coy laboratory Products inc., Michigan, USA) at 35 °C. Higher incubation temperatures (although favorable for growth) in the glove box resulted in evaporation of the water from the plates and condensation on the chamber walls. The enrichment cultures were also incubated at 35 °C. Test tubes and all other sealed vials as well as plates closed up in Anaerocult A bags (Merck, Darmstadt, Germany) were incubated at 37 °C in a thermostated chamber or in incubators.

7.5. Degradation tests with mixed cultures

7.5.1. Weight loss determination

Anaerobic microbial attack in the cultures containing each three pre-weighed and sterile polyester films (see table 7.2) supplemented with anaerobic sludges and a sediment (see 7.2.1.) was determined by measuring the weight loss of polyester films after the appropriate incubation period. After recovery, the polyester samples were washed twice with distilled water, dried to constant mass under vacuum and reweighed. The mean weight difference of the films (at least triplicates) was expressed as weight loss (Δm in mg) or optionally expressed as $\Delta m\ A^{-1}$ in mg cm^{-2} (A = total surface area of the polyester strip in cm^2, see table 7.2) since polymer depolymerization is a surface process.

7.5.2. Biogasification as indicator for polyester mineralization

Monitoring of biogas production for the quantitative comparison of anaerobic degradation with mixed cultures was done as described by (PÜCHNER, 1995) with undiluted and diluted

sludges according to ASTM D 5210-91 (1991). The biogas produced in sealed vials displaces an equal volume of a liquid (20 % (w/v) NaCl and 0.5 % (w/v) citric acid) in a graduated gas collecting tube (25 ml) directly connected to the vials thereby allowing the vials to remain at atmospheric pressure throughout the assay (no changes in gas solubility resulting from pressure build-up in the vials).

For these experiments undiluted and diluted anaerobic sludges were used as inoculum according to DIN 38414 S8 (1985). The test set up containing each one polyester film (table 7.2) was thermostated in a chamber with a constant temperature of 37 °C. To avoid measurements of additional gas production caused by digestible organic materials in the inoculum, the sludge was incubated for several days prior to inoculation until gas evolution was not observed for at least one week. In addition, the volumes of biogas produced by the blank vessels (mean of two samples of digested sludge without any added substrate) were subtracted from the values obtained for the individual duplicate test set-ups.

The percentage degradation (%ThBiogas) was determined as the ratio of the cumulative net gas production to the theoretical value calculated from the C-content of the sample (table 7.4) based on the Buswell-equation (BUSWELL AND MÜLLER, 1952):

$$C_nH_aO_b + (n - a/4 - b/2)H_2O \rightarrow (n/2 - a/8 + b/4)CO_2 + (n/2 + a/8 - b/4)CH_4$$

Biogas production was measured at two to three days intervals over a test period of at least 42 days and the cumulative gas production (biogas from the test vials minus biogas from the control vials, and corrected for temperature and pressure) converted into % ThBiogas was plotted versus incubation time.

Table 7.4. The theoretical biogas production for each polyester.

Polyester	Emperical formula	Moles of biogas produced	C-content (Weight %)	ThBiogas ($l\ g^{-1}$)
PHB	$(C_4H_6O_2)_n$	4	55.8	1.04
PHBV	$((C_4H_6O_2)_l\ (C_5H_8O_2)_m)_n$	4.1	56.4	1.05
PCL	$(C_6H_{10}O_2)_n$	6	63.1	1.18
SP 4/6	$(C_{10}H_{16}O_4)_n$	10	60.0	1.12
SP 3/6	$(C_9H_{14}O_4)_n$	9	53.5	1.0
BTA 40/60	$((C_{10}H_{16}O_4)_l\ (C_{12}H_{12}O_4)_m\ )_n$	10.8	61.4	1.15

7.6. Screening and isolation procedures of polyester depolymerizing anaerobes

7.6.1. Enrichment cultures

Each of the three mesophilic sludges (***WWS, LS, AS,*** see 7.2.1.) was supplemented with one of the six polyesters (see 7.1, fig. 7.1) and optionally with all six polyesters at a time. Using the different enrichment cultures of the unidentified consortia (sludges and sediment) in which polyester strips were incubated for 14 weeks and optionally for 18 month potential polyester depolymerizing strains were screened.

7.6.2. Assessment of enrichment

Using the polyester containing agar plates and roll tubes described in 7.2.3. and 7.3 the presence of an enriched population of polyester depolymerizing anaerobes in the various enrichment cultures was checked after 14 weeks and optionally after 18 month of incubation at 35 °C. The enriched microbial population containing the potential polyester depolymerizing organisms was cultivated on rich complex media (see 7.2.3, table 7.3, ***TVLS***, ***Br*** and ***TG***) leading to high cell densities, and large as well as cultivable colonies. Then, morphologically different colonies were subcultured on polyester containing mineral salt agar plates and potential depolymerizing anaerobes were selected via clear zone formation.

7.6.3. Replica plate technique

In an additional step, the replica plating technique was applied. The potential depolymerizing organisms were inoculated parallel on different rich media and on MSV media containing one of the following substrates as a co-metabolite such as yeast extract (Serva, New York, USA), glucose, sodium acetate, sodium tri-citrate (Merck, Darmstadt, Germany) and sodium crotonate (Sigma, St. Louis, USA) at a final concentration of 0.1 % (w/v). Hence, the individual strains were tested for their ability to grow on and depolymerize the polyesters incorporated in the mineral-salt-vitamin-(MSV) agar plates supplemented with and without different co-substrates. The selection criterion was the ability to form clear zones.

7.6.4. Purification of polyester depolymerizing strains

Positive strains forming colonies surrounded by clear zones after incubation for 15 days at 35 °C were isolated by picking the colonies using sterile tooth picks and further purified on complex media (see 7.2.3, table 7.3) using the standard spatial streaking method on solid agar media plates and preserved on rich complex media. All inoculations and incubations

were performed in an anaerobic glove-box (Coy laboratory Products inc., Michigan, USA) filled with 95 % N_2 and 5 % H_2 as head space gas at 35 °C.

7.6.5. Preservation

The isolates were preserved in airtight vials containing 50 % (v v^{-1}) glycerol (87 %) flushed and head-space filled with oxygen free N_2-gas. The anaerobic vials were additionally put in air tight bags containing an anaerobic catalyst (Anaerocult A, Merck, Darmstadt, Germany) and stored at -20 °C.

7.7. Identification of the isolated strains

7.7.1. DNA base composition and 16S rDNA partial sequence analysis

The G+C mol % content was analyzed using the HPLC method of MESHBAH ET AL. (1989). The 16S rDNA partial sequence analysis is based on the determination of parts of the 16 S rDNA nucleotide sequence via direct sequencing of the 16S rDNA and amplifying each part of the genomic DNA using PCR. For the extraction of the genomic DNA, the PCR amplification of the 16S rDNA and the purification of of the PCR products the methods described by RAINEY ET AL., 1996 were used. Purified PCR products were sequenced with *Taq* Dyedeoxy terminator cycle sequencing kits (Applied Biosystems, Weiterstadt, Germany) as directed in the manufacturer's protocol. The resulting sequence data were interpreted according to MAIDAK ET AL. (1996) and compared with the 16S rDNA nucleotide sequences of representative organisms of the main bacterial lineages available from public databases (MAIDAK ET AL., 1996).

7.7.2. Biochemical characterization of the isolates

The selected isolates were chracterized according to the standard methods described by HOLDEMANN AND MOORE (1978) as well as KRIEG (1981) and were partially performed by the DSMZ, Germany.

7.8. Microscopic examinations

7.8.1. Light microscopy

For purification examination and morphological investigations native samples the different strains were studied using the phase contrast light microscope (Axioscop, Zeiss, Oberkochen, Germany).

The macroscopic appearance and culture morphology on solid agar media as well as polyester film surfaces were studied using a Stemi 2000 microscope (Zeiss, Jena, Germany) supplemented with a camera (Minolta EX 300 Reflex).

7.8.2. Scanning electron microscopy (SEM)

The surface microstructure of polyester films as well as changes due to degradation were examined using a DSM 982 Gemini (Zeiss, Oberkochen). The washed and dried polyester samples were coated with gold under an argon atmospere at a distance of 50 mm at 45 mA 50 s (Sputter-Gerät SCD 040, Bal-Tec, Liechtenstein). The examinations were performed by Dr. H. Lünsdorf (GBF, Braunschweig).

7.9. Degradation tests with isolated strains

7.9.1. Polyester depolymerization measured by clear zone formation

For clear zone tests (AUGUSTA ET AL., 1993) turbid MSV agar plates (see 7.2.3.) containing the polyester under investigation were inoculated with colonies from agar plates either with sterile toothpicks or inoculation needles or from liquid cultures by spreading the liquid inoculum using a glass triangle. The increase in clear zone diameters developing on the MSV agar plates was followed up periodically and measured with a slide gauge.

7.9.2. Polyester hydrolysis in liquid culture

Sterile circular polyester films (Table 7.2) were added to Hungate tubes containing sterile MSV medium and inoculated with 100 µl of a suspension of the isolate (OD_{600} = 0.8, TG medium). The degradation of films was examined after incubation for the desired incubation period at 37 °C by weight loss determination as described under 7.5.1.

7.9.3. Polyester hydrolysis via agar plate method

As an indication for polyester depolymerization the weight loss of polyester films laid on agar plates and inoculated with the purified and characterized *Clostridium* strain 5a was studied. Sterile circular films (25 mm diameter, 100 µm thickness, triplicates; (m_i = 26 – 40 mg); degradation surface area: 19,6 cm^2; table 7.2) of PHB and PHBV were incubated on MSV medium supplemented with 0.1 % (w v^{-1}) yeast extract. The seed culture was prepared in *TG* liquid medium (table 7.2) in “Hungate tubes” inoculated with the strain under investigation and shacked in a rotary shaker at 150 rpm and 37 °C at an angle of about 65 ° until an OD_{600} = 0.8 was obtained. The degradation test tubes were inoculated with 100 µl from this seed culture. Degradation of the PHB and PHBV films was examined after

incubation for 11 weeks at 35 °C in a an anaerobic glove-box (Coy laboratory Products inc., Michigan, USA) filled with 95 % N_2 and 5 % H_2 as head space gas at 35 °C by weight loss determination as described under 7.5.1. Sterile incubated controls incubated over the same period were performed and showed no abiotic hydrolysis of the polyester samples.

7.10. Degradation test in a controlled bioreactor

The cultivation of the biomass and the degradation test were carried out in a one liter stirred tank bioreactor “BIOSTAT Q” (B.Braun Biotech International, Germany) with a working volume of 650 ml. The reactor was equipped with pH, temperature and agitation speed controllers. The bioreactor was gassed with 100 % oxygen free nitrogen gas via a fine porous gas distributor. (Thermal mass flow meters/controllers were used). The pH was controlled at 6.8 and the agitation speed was adjusted to 150 rpm during the degradation run. The inoculum was set to contribute to 10 % (v v^{-1}) of the working volume. The degradation experiments were performed at 37 °C.

7.11. Determination of PHB degradation

Degradation of PHB in the bioreactor was determined by the method of SENIOR ET AL. (1972). A sample of the culture broth (0.01 – 0.5 ml) was added to 9 ml of a 10 % alkaline hypochlorite solution and incubated at 20 °C for 24 h. After centrifugation at 5500 g for 45 min the PHB granules were separated and the supernatant decanted. The solid pellet was resuspended and washed successively with 10 ml portions of water, acetone and ether. After drying (40 °C for 24 h) the white powder was dissolved in conc. H_2SO_4 (10 ml) and heated for 10 min at 100 °C. After cooling the solution was read at 235 nm against a conc. H_2SO_4 blank. A calibration curve was constructed with PHB powder. Protein concentration was determined in the supernatant by the method of LOWRY ET AL. (1951).

7.12. Analytical methods

7.12.1. Gel permeation chromatography (GPC)

The molecular weight distribution of the polymeric materials was determined using a Techlab chromatograph equipped with a pre-column (Plgel®, 5 guard, 50 x 7.8 mm, Latek, Eppenheim, Germany) and a separation column (PLgel®, 300 x 7.5 mm (5 x 10^4), Latek, Eppenheim, Germany) with a functional molecular weight range of 11 800-500 000 g mol^{-1}.

Aliquots of 100 µl of the sample under investigation were previously dissolved in di-chloromethane and injected. Chloroform (HPLC grade, degassed and filtered) was used as the mobile phase at a flow rate of 1.0 ml min^{-1} at room temperature. The GPC unit is equipped with a refractive index (RI) detector (SKD Shodex RI SE-51) and a UV-detector (Gynkotek SP-6 V).

Number-average (M_n) and weight-average (M_w) molecular weights were calculated relative to polystyrol standards (Software: Chromostar, Bruker).

7.12.2. Determination of the relative starch content of the blended polyesters

The relative starch content of the blended polyesters was determined to follow up the changes with the proceeding of the degradation. Two different methods were performed: first, gravimetrically and second by applying GPC.

a-Gravimetric determination of the starch content

A pre-weighed circular polyester film (Ø 38 mm) was dissolved in 1 ml di-chloromethane in a pre-weighed 1 ml test vial (Eppendorf). After centrifugation for 15 min at 15 000 rpm, the supernatant was carefully removed and the remaining pellet dissolved again in 1 ml of di-chloromethane. These washing and centrifugation steps were repeated three times for each sample. Finally, the cups were dried under vacuum at 37 °C for 36 hours and re-weighed. The differences in weight between the polyester weight and the starch pellet weight allows the calculation of the starch content.

To estimate possible weight losses due to the dissolution of the cup-material due to di-chloromethane treatment, the cups were treated the same way as mentioned above without polyester samples. An increase in weight accounting to 0.2 % was noticed.

b-Determination of the starch content by GPC

Comparing the polyester content of pure polyester films to that of the starch containing blends allows the calculation of the starch content of the blended materials. Calibration curves of pure PCL and BTA were established in a concentration range of 0.25 – 1.5 mg l^{-1} in chloroform (R^2 for PCL: 0.9997; R^2 for BTA: 0.9996). For analysis of starch content the blend was dissolved in chloroform to a known concentration. The residual solid starch was thereafter separated by filtration (0.8 and 0.45 µm filter paper) and the polyester containing solvent was analyzed by GPC and the concentrations calculate from the GPC-peak areas.

7.12.3. Determination of the optical density

The optical density was measured at 600 nm using a Ultrospec 1000 spectrophotometer (PharmaziaBiotech, Freiburg, Germany).

a-Determination of the optical density of fermentation broth

From the fermentation broth aliquots of 1 ml were measured in triplicates. If the extinction was higher than 0.4 appropriate dilutions (three different dilution factors) were performed in 1 ml cuvettes.

b-Determination of the optical density in "Hungate" tubes

The "Hungate" tubes were cleaned with 70 % ethanol, wiped with a clean tissue, and mixed thoroughly with a vortex (Vortex Genie 2^{TM}, Bender & Hobein AG, Zürich, Swiss) for 60 seconds. The tubes were marked at a certain position to measure each tube each time at the same position to exclude differences according to scratches on the glass wall of the tubes.

7.12.4.Preparation of buffers

Table 7.5. lists the different buffer systems used throughout the present work and their applications.

Table 7.5. Buffer systems and their applications.

Buffer	pH-range	Molarity	Application
Phosphate buffer: KH_2PO_4 / Na_2HPO_4	6.8 ± 0.1 6.8 ± 0.1	20 mM 50 mM	Enzyme purification (phenyl sepharose) Enzyme elution from PHB pellet
Glycine buffer: Glycine+NaCl / HCL 0.1 M	8.6 – 12.8	0.1 M	Determination of pH optimum of the purified enzyme
Borate buffer: Boric acid / HCL 0.1 M	7.8 – 9.2	50 mM	Determination of pH optimum of the purified enzyme
Citrate buffer: Citric acid / Na_2HPO_4 20 mM	2.2 – 7.8	0.1 M	Determination of pH optimum of the purified enzyme

7.12.5. Determination of Protein content

The protein concentration of cells and enzyme samples was determined according to the method by LOWRY (1951) using bovine serum albumin as standard protein.

a-Determination of the protein content of cells in pellet

When the protein content of cells mixed with PHB granules was to be determined, the bacterial cells were first digested and afterwards the protein content was determined. For this purpose the sample pellet (cells + PHB) was suspended in 300 ml of 1 M NaOH and incubated for 1 hour at 75 °C in a thermo-mixer (5436, Eppendorf, Hamburg). The mixture was then centrifuged at 14 000 rpm for 10 min and the pellet consisting of cell derbies and the rest PHB was discarded. 100 µl of the supernatant were used for protein determination as described by LOWRY (1951). The standard curve prepared with bovine serum albumin was also treated with 1 M NaOH.

b-Determination of soluble proteins

Since all anaerobic media contain reducing agents which negatively interfere with this method, proteins in solution were precipitated first with trichloracetic acid, centrifuged, and re-suspended in an equal amount of Milli Q water before the addition of reagents (PETERSON, 1977).

7.12.6. Gas chromatographic methods for the determination of fermentation end products

The fermentation end products were analyzed by gas chromatography using a Chromopack (Model 438 A, Chromopack, Frankfurt, FRG) equipped with a glass column of 1 m length filled with Chromosorb 101 (100 – 200 mesh) over a flame ionization detector (T = 250 °C). N_2 was the carrier gas, the oven temperature was programmed between 150 and 200 °C. For analysis 1 ml culture samples were centrifuged 10 minutes at 14 000 rpm and the supernatants if required diluted to the required concentration with Mili Q water.

A two point calibration was performed using the standards as described below and was repeated after measuring 4 samples. An internal standard consisting of 1.4 % n-butanol in 1 M HCL was added to the standards as well as the samples at an concentration of 10 %. Each undiluted sample was measured three times. Concentrated samples were diluted twice by the same dilution factor and each sample was measured three times, in order to compensate dilution errors.

The composition of the standard solutions is as follows:

Component:	***Standard 1:1 (mmol/l)***	***Standard 1:2 (mmol/l)***
Ethanol	10	5
Acetate	25	12.5
Butyrate	25	12.5
1,3-propanediol	50	25

Sample volumes of 1 µl were injected automatically using an auto-sampler (Type LS 607, Packard, Zürich, Swiss). The chromatograph was directly connected with a Chromopack Integrator (Modell C-R2A, Shimadzu, Tokyo, IPN). The interpretation of data was done with the Apex Chromatography Workstation (Autochrom Incorporated, POB 207, Milford, MA 01757, USA).

7.12.7. Enzyme test

a-Preparation of the stable PHB suspension

For the preparation of a stable PHB suspension 1.2 g PHB powder were suspended in 59 ml MSV-medium (table 7.3) and soniccated for 7 minutes at 90 duty cycles (Branson Sonifier; Branson Ultrasonic Cooperation, Danburry, CT, USA). Afterwards the suspension was left to settle down and after 5 minutes the supernatant was separated. 1ml of the supernatant was transferred into 500 ml fresh MSV medium, flushed with N_2, pH adjusted to 7 and closed air tight. After autoclaving, the suspension was supplemented with reducing agents, vitamins and the additives normally included in the medium (table 7.3). The pH wais readjusted to 6.8 using sterile 1 M NaOH and 1 M HCL. Thereafter, 4 ml of this sterile suspension were transferred via sterile syringes into anaerobic N_2- flushed "Hungate tubes" which have been previously sterilized. Tubes showing the characteristic pink color of the oxidized resazurin were discarded.

b-Measuring enzyme activity

The applied test depends on mixing the sterile filtered culture supernatant with a stable PHB suspension prepared in the reduced medium (normally applied for cultivation) in anaerobic "Hungate" tubes. After determination of the initial OD_{600nm} of this suspension-crude enzyme mixture, it was incubated with constant agitation (150 rpm) at 37 °C for at least 24 hours. The decrease of the optical density of this suspension with time allowed tracing of enzyme (production) activity and the calculation of PHB degradation using a standard curve (see appendix fig. 9.4).

7.12.8. Enzyme purification

7.12.8.1. Ultrafiltration

In order to concentrate enzyme containing solutions ultrafiltration units (Model 8050, Amicon, Beverly, MA, USA) were used. The ultrafiltration membrane consisted of regenerated cellulose (UF-Membran RC, Membrapure GmbH, Bodenheim, Germany) with a cut off of 10 and later of 30 kDa and a diameter of 47 mm. The ultrafiltration process was

carried out with N_2 gas at 3 bar pressure in a cooling room at 4 °C. After usage, the ultrafiltration membrane was washed with 0.1 N NaOH followed with destilled water and stored at 4 °C in a 10 % (v v^{-1})ethanol solution. Optinally, ultrafiltration was carried out using Omega^{TM} unit (Pall-GelmanSciences, Dreieich, Germany) with a cut off of 100 kDa (ultrafiltration membrane: modified polysulfon for reduced protein adsorption; volume: 150 ml; maximal concentration: 10 %; maximl pressure: 3.7 bar) and a diameter of 60 mm. The ultrafiltration process was carried out with N_2 gas at 3 bar pressure in a cooling room at 4 °C. After usage, the ultrafiltration membrane was washed with 0.1 – 1.0 N NaOH followed with destilled water and stored at 4 °C in a 70 % (v v^{-1}) ethanol solution.

7.12.8.2. Dialysis

Dialysis was carried out for 12 – 24 hours at 4 °C in dialysis tubings (Spectra/Por® MWCO: 6000-8000, Ø 2.55 cm, Spectrum Medical Industries, INC., Laguna Hills, CA, USA) which were closed at both ends with clamps using 100 fold bigger volumes. The buffer was changed every 4 – 8 hours. Salt concentration was checked by conductivity measurements (Conductivity Meter LF 318, WTW, Weilheim, Germany).

7.12.8.3. Fast Protein Liquid Chromatography (FPLC)

Trials to purify the target enzyme via HIC chromatography was performed using a standard FPLC unit (LCC-500 Plus) equipped with automatic equilibration, injection and elution facilities (Pharmacia, Uppsala, Sweden) at room temperature.

a-Chromatographic materials

The chromatographic materials used are listed in Table 7.6.

Table 7.6. Materials used for hydrophobic interaction chromatography.

Chromatographic material	Co.-Number	Source
Phenyl sepharose CL-4B	17-0810-01	Fluka, Deisenhofen, Germany
HITrap® HIC Test kit:	17-1349-01	Pharmacia Biotech, Freiburg, Germany
Phenyl sepharose high performance		
Phenyl sepharose high performance		
Phenyl sepharose 6 fast flow (high sub)		
Phenyl sepharose 6 fast flow (low sub)		
Butyl sepharose 4 fast flow		
Octyl sepharose 4 fast flow		

b-Buffer and sample preparation

Buffers and samples were filtered (RC 58 membrane filter, 0.2 µm, Ø 47 mm, Scleier & Schüll, Dassel, Germany) and de-gassed (30 min under continuos stirring with a membrane vacuum pump equipped with a vacuum controller, (Vaccumbrand, Wertheim, Germany)) prior to the application to the FPLC unit to prevent clogging of and air bubble formation in the columns.

c-Determination of salt concentration

Using the "test tube method" described by PHARMACIA (1997) the minimal concentration of ammonium sulfate allowing the binding of the target enzyme to phenyl sepharose CL-B4 was determined in a batch test (test tubes, 1ml). The ammonium sulphate concentration in a 20 mM phosphate buffer was varried as follows: 0, 0.5, 0.75, 1, 1.2, 1.5 M.

7.12.9. Analytical SDS gel electrophoresis

7.12.9.1. Sample preparation

In order to concentrate the protein samples containing the target enzyme for gel electrophoretic investigations 2 ml of the enzyme sample in 2 ml tubes (Eppendorf) were treated with 20 µl StrataClean^{TM} resin (No. 400714, Stratagene, Amsterdam, Netherlands) and then incubated for 20 minutes in a thermo-mixer (5436, Eppendorf, Hamburg) under continuous shaking at room temperature. Thereafter the enzyme samples were centrifuged at 14 000 rpm for 6 minutes and the pellet was re-suspended in 20 µl sample buffer (table 7.6) and heated at 95 °C for 6 minutes in a thermo-mixer (5436, Eppendorf, Hamburg). Samples treated this way can be stored at –20 °C.

7.12.9.2. SDS-PAGE

SDS gel electrophoresis was carried out by using 8 - 18 gradient EXELGELS SDS (Pharmacia Biotech, Freiburg, Germany) using a Multiphor II gel electrophoresis system (Pharmacia Biotech, Uppsala, Sweden). The samples were treated according to the producer instructions (Instructions, 80-1310-00 Edition AE, Pharmacia Biotech, Freiburg) with non reducing sample buffer A. The sample volume ranged from 15 to 20 ml applied on applicators laying on the gel. Separation of the proteins was performed at 15 °C, 600 V, 50 mA and 30 W over a period of 75 minutes. Optionally, proteins were separated by denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis by the method described by LAEMMLI (1970) using a Biometra Multigel long electrophoretic unit (Biometra, Göttingen, Germany) and an electrophoresis Power supply (EPS 601, Pharmacia Biotech,

Freiburg, Germany). See table 7.7 and 7.8 for the composition of the electrophoretic buffers and gels.

Table 7.7. Composition of the buffers required for SDS-PAGE.

Sample buffer	Electophoretic buffer	4x Lower Tris	4x Upper Tris
100 mM Tris HCL, pH 6.8	30.3 g l^{-1} Tris base	181.7 g l^{-1} Tris base	66.55 g l^{-1} Tris base
200 mM DTT freshly prepared	144.0 g l^{-1} Glycine	4 g l^{-1} SDS	4 g l^{-1} SDS
4 % SDS	10 g l^{-1} SDS	**pH 8.8 with HCL**	**pH 6.8 with HCL**
0.2 % Bromophenol blue			
20 % Glycerin			

Table 7.8. Composition of the running gels for SDS-PAGE (gel thickness 1 mm)

Solution	Running gel final concentration		
	4 %	15 %	18 %
30 % Acrylamide / 0.8 % N,N-Methylenbisacrylamide	4.6 ml	49.5 ml	40.5 ml
4 x Upper tris	8.25 ml	-	-
4 x Lower Tris	-	25.1 ml	17.1 ml
ddH_2O	19.8 ml	23.8 ml	11.25 ml
APS, 10 %	79.2 µl	119 µl	81 µl
TEMED	39.6 µl	79 µl	54 ml

Proteins from a molecular weight calibration kit (Pharmacia Biotech, Freiburg, Germany) or 10 kDa protein ladder (Cat. No. 10064-012, Life Technologies TECH-LINE^{SM}) were used as M_r standards. After electrophoresis, proteins were silver stained using a "silver staining kit" (Code No. 17-1150-01, Pharmacia Biotech, Freiburg, Germany) or according to MERRIL (1984).

7.12.9.2. Native gel electrophoresis

Under native conditions, separation of proteins depends on many factors including size, shape and native charge. One straight foreword approach to native gel electrophoresis is to leave out the SDS and the reducing agent (DTT) from the standard Laemmli SDS PAGE protocol. Thus, the sample buffer contains neither SDS nor DTT (see table 7.9 and 7.10),

samples are not heated and the gel and electrode solutions are prepared without SDS (HAMES, 1990).

Table 7.9. Composition of the native sample- and running buffers.

Native sample buffer	Running gel buffer
62.5 mM Tris, pH 6.8	25 mM Tris, pH 8.6
30 % (v/v) Glycerin (100 %)	192 mM Glycin
0.25 g l^{-1} bromophenol blue	Prepared as 10 fold stock buffer, 4 °C.

Table 7.10. Composition of the stacking and running gels for native gel electrophoresis (gel thickness 1 mm).

Solution	Stacking gel	Running gel
ddH_2O	3.2 ml	7.2 ml
Tris, 1.5 M, pH 8.8	-	3.8 ml
Tris, 0.5 M, pH 6.8	1.25 ml	-
Bis/acrylamide solution	0.5 ml	4 ml
300g l^{-1} / acrylamide / 8 g l^{-1} N,N-Methylenbisacrylamide		
TEMED	15 µl	20 µl
APS, 10 %	60 µl	120 µl

Proteins from a high molecular weight calibration kit for native electrophoresis (Amerscham Pharmacia Biotech, Freiburg, Germany) were used as M_r standards. After electrophoresis, proteins were silver stained using a "silver staining kit" (Code No. 17-1150-01, Pharmacia Biotech, Freiburg, Germany) or according to MERRIL (1984).

In order to determine the active PHB depolymerizing protein band the active enzyme sample was diluted in a ratio of 1:2 with the native sample buffer and applied on the gels. After running two identical native gels with an active enzyme sample one whole gel was stained (silver stain) to determine the position of the separated protein bands and its replica was immersed in reduced MSV medium and incubated anaerobically at 55 °C for at least 48 hours for activity testing.

7.13. Chemicals and apparatuses

The source of chemicals and the different apparatuses are listed in table 7.11 and 7.12, respectively.

Table 7.11. List of chemicals used throughout this work.

Chemicals	Source
Salts, acids, alkalis, etc.	Merck, Darmstadt, Germany,
with a purity degree of > 95 %.	Riedel de Häen, Seelze, Germany,
	Fluka, Deisenhofen, Germany,
	Sigma, Deisenhofen, Germany
Bis-acrylamide solution	Roth, Karlsruhe, Germany
Bobvine serum albumin	Sigma, Deisenhofen, Germany
Cultivation media	Merck, Merck, Darmstadt, Germany
EXELGELS SDS	Pharmacia Biotech, Freiburg, Germany
High molecular weight calibration kit	Amerscham Pharmacia Biotech, Freiburg, Germany
Low molecular weight standard	BioRad, München, Germany
Molecular weight calibration kit	Pharmacia Biotech, Freiburg, Germany
10 kDa protein ladder	Life Technologies TECH-LINE^{SM}, Karlsruhe, Germany.
Phenyl sepharose CL-4B	Fluka, Deisenhofen, Germany
Polyesters	See table 7.1
Silver staining kit	Pharmacia Biotech, Freiburg, Germany

Table 7.12. List of apparatuses used throughout this work.

Apparatus	Source
Incubator UE 700	Memmert, Schwabach, Germany
Millipore-Q-unit	Millipore, Eschborn, Germany
Multiphor II gel electrophoresis system	Pharmacia Biotech, Uppsala, Sweden
Shaking incubator	Infors HAT®, Bottningen, Germany.
Thermomixer 5436	Eppendorf, Hamburg, Germany
Vacuum incubator VT 5042 EK	Heraeus Holding GmbH, Hannover, Germany
Vortex Genie 2^{TM}	Bender & Hobein AG, Zürich, Swiss.
Centrifuges:	
Eppendorf-Centrifuge 5415	Eppendorf, Hamburg, Germany
Sorvall T6000B	Sorvall GmbH, bad Homburg, Germany
Suporafuge 22	Heraeus holding GmbH, hannover, Germny

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9. Appendix

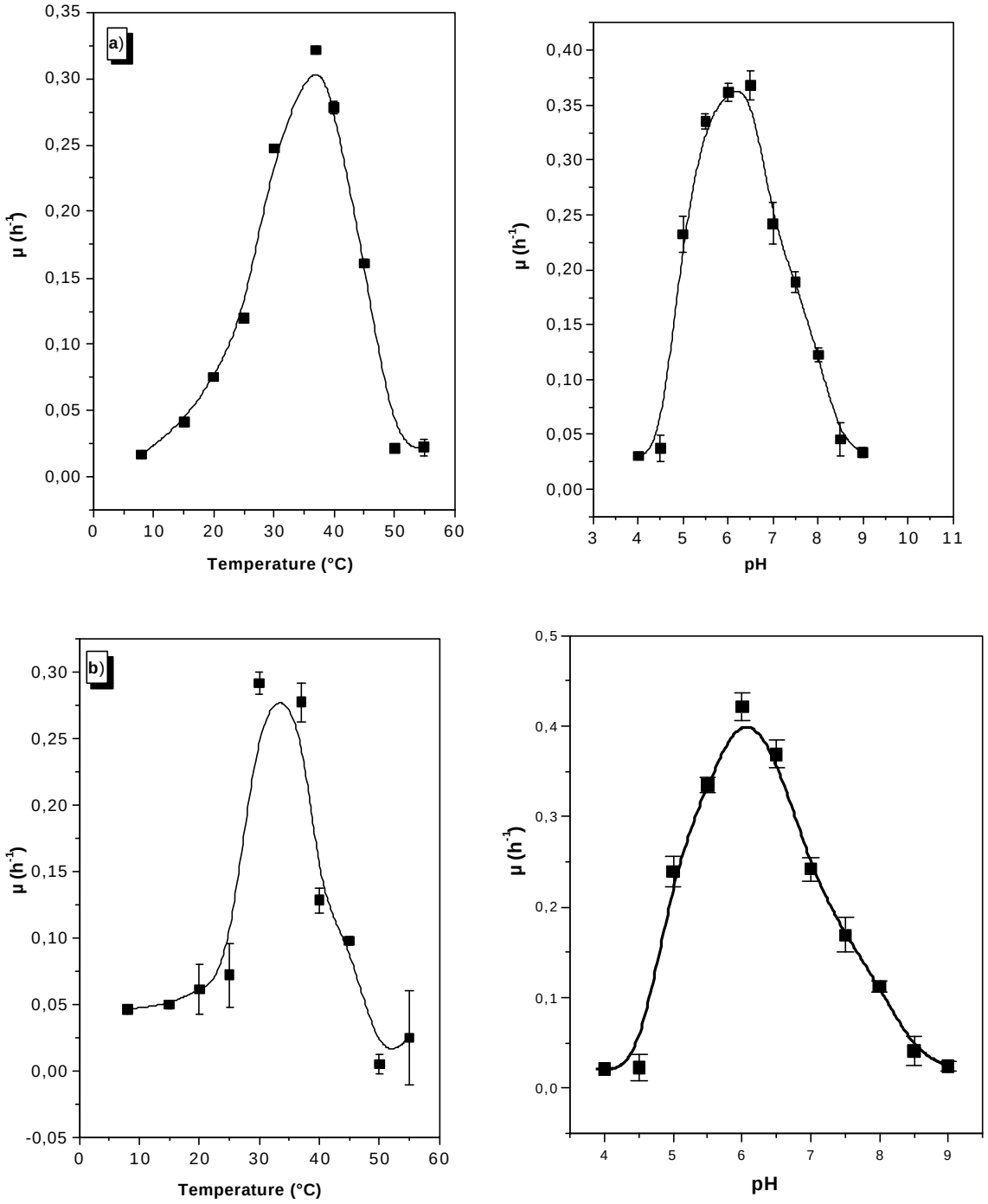

Fig. 9.1. Specific growth rates as function of incubation temperature and pH-value at 37 °C of (a) strain 5a and (b) strain Cont b.

Table 9.1. Phenotypic and biochemical characters of isolated strain 5a.

Character	*Strain 5a*
Cell shape	rod shape
Spore shape	oval
position	Sub-terminal; terminal
Swollen sporangium	+
Cell size	0.54-0.9 x 2.25-6.3µm
Motility	+
Growth in PY	+
in PYG	+
Gas production	-
Gelatin hydrolysis	-
Meat degrdation	nd
Indole production	-
Lecithinase	-
Lipase	-
Nitrate reduction	-
AMC-production	nd
Esculin hydrolysis	-
Fermentation of carbohydrates:	
Amygdalin	-
Arabinose	-
Cellobiose	-
Esculin	-
Fructose	-
Galactose	-
Glucose	-
Glycogen	-
Inosit	-
Inolin	nd
Lactose	-
Maltose	-
Mannit	-
Mannose	-
Melezitose	-
Melibiose	-
Pectin	nd
Raffinose	-
Rhamnose	-
Ribose	-
Salicin	-
Sorbit	-
Starch	-
Saccarose	-
Trehalose	-
Xylose	-
Lactate	+w
Pyruvate	-

+: positive; w: weakly positive; -: negative; +/-: most strains are positive; nd: not detected; ST: sub-terminat; t: terminal; AMC: acetylmethylcarbinol; c: coagulate; a: acid; g: gas; ab: milk protein degradation; PY: peptone-yeast extract medium; PYG: peptone-yeast extract medium with 1 % glucose.

Table 9.2. Phenotypic and biochemical characters of isolated strains PCL 6 and PCL 7 (AFTER HOLDEMANN ET AL., 1978; BERGEY´S MANUAL, 1986).

Character	***PCL 6***	***PCL 7***
Gram stain reaction	- (GMB; young)	- (GMB; young)
Cell shape	rod shape	rod shape
Cell size	0.68-0.9µm x 3.6-6.8 (9) µm; filaments (31.5 µm)	0.9 µm x 3.0-10.4 µm
Spore shape	oval	oval
position	Sub-terminal; terminal	Sub-terminal; terminal
Swollen sporangium	+/-	+/-
Cell size	nd	nd
Motility	w	w
Growth temperature	37 °C	37 °C
Growth in PY	+	+
in PYG	+++	+++
Gas production	+	+
Gelatin hydrolysis	w	w
Meat degrdation	nd	nd
Indole production	-	-
Lecithinase	-	-
Lipase	-	-
Nitrate reduction	-	-
AMC-production	nd	nd
Esculin hydrolysis	+	+
Milk reaction	c, g, a	c, g, a
Fermentation of carbohydrates:		
Amygdalin	+	+
Arabinose	+	+
Cellobiose	+	+
Esculin	+	+
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycogen	+	+
Inosit	-	-
Inolin	nd	nd
Lactose	+	+
Maltose	+	+
Mannit	+	+
Mannose	+	+
Melezitose	-	-
Melibiose	+	+
Raffinose	+	+
Rhamnose	+	+
Ribose	+	+
Salicin	+	+
Sorbit	-	-
Starch	+	+
Sucrose	+	+
Trehalose	+	+
Xylose	-	-

Abreviations see table 1.

Table 9.3. Phenotypic differences between *C. acetobutylicum* and the isolated strain PCL 6. (abbreviations see Table 9.1).

Character	*PCL 6*	*C. acetobutylicum*
Gram stain reaction	- (GMB; young)	+
Cell shape	rod shape	rod shape
Cell size	0.68-0.9 µm x 3.6-6.8 (9) µm; filaments (31.5µm)	0.5 – 0.9 µm x 1.6 – 6.4 µm
Spore shape	oval	oval
position	Sub-terminal; terminal	Sub-terminal
Swollen sporangium	+/-	+w
Motility	w	+
Growth temperature	37°C	37 °C
Growth in PY	+	+
in PYG	+++	+++
Gas production	+	+
Gelatin hydrolysis	w	-/+w
Meat degrdation	nd	-
Indole production	-	-
Lecithinase	-	-
Lipase	-	-
Nitrate reduction	-	-
AMC-production	nd	+
Esculin hydrolysis	+	+
Milk reaction	c, g, a	c, g, a
Fermentation of carbohydrates:		
Amygdalin	+	-
Arabinose	+	nd
Cellobiose	+	+
Esculin	+	-
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycogen	+	nd
Inosit	-	-
Inolin	nd	nd
Lactose	+	+
Maltose	+	-/+
Mannit	+	+
Mannose	+	-
Melezitose	-	-
Melibiose	+	-
Raffinose	+	-
Rhamnose	+	-
Ribose	+	+
Salicin	+	+
Sorbit	-	-
Starch	+	+
Sucrose	+	+
Trehalose	+	-/+
Xylose	-	nd

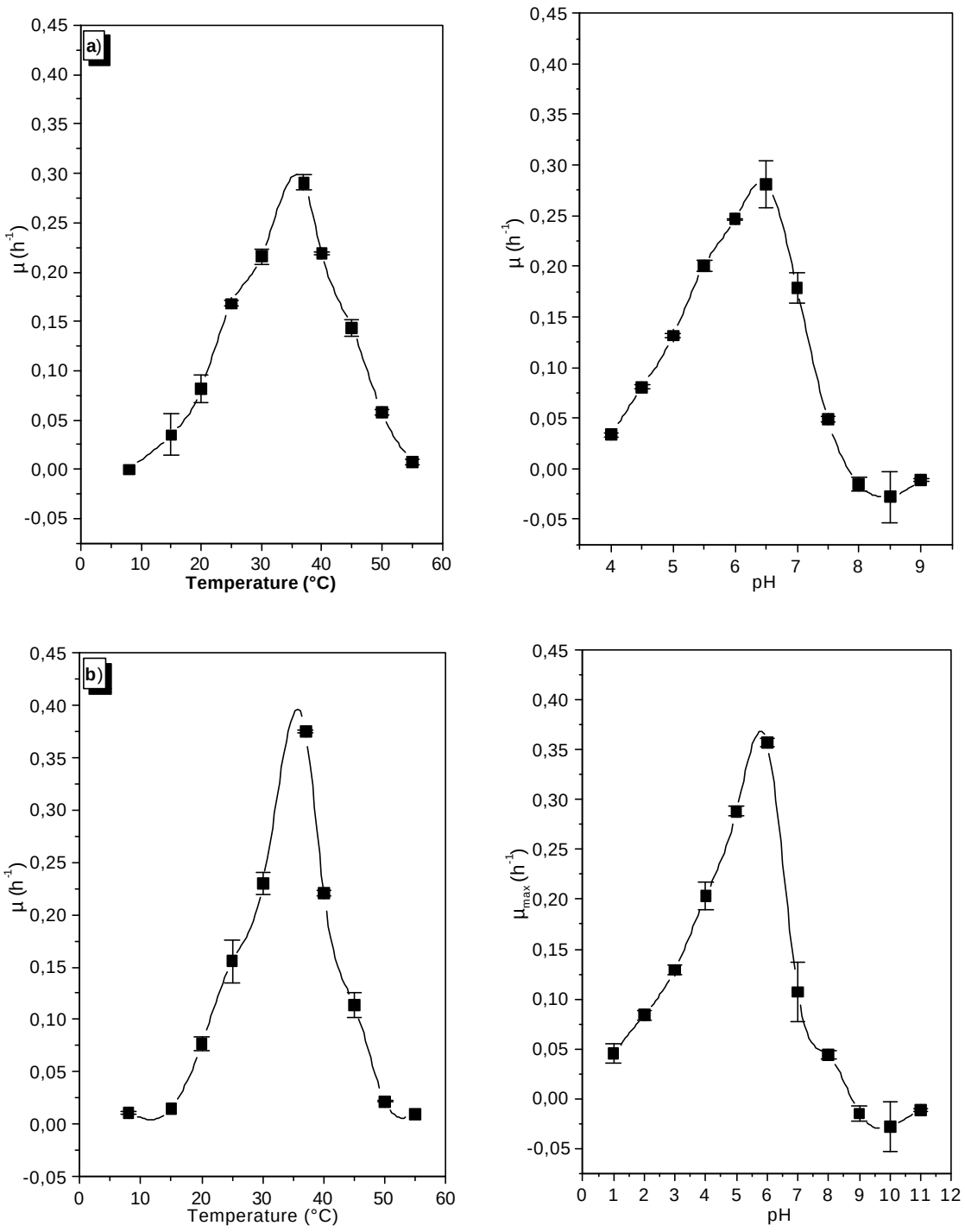

Fig. 9.2. Specific growth rates as function of incubation temperature and pH-value at 37 °C of (a) strain PCL 6 and (b) strain PCL 7.

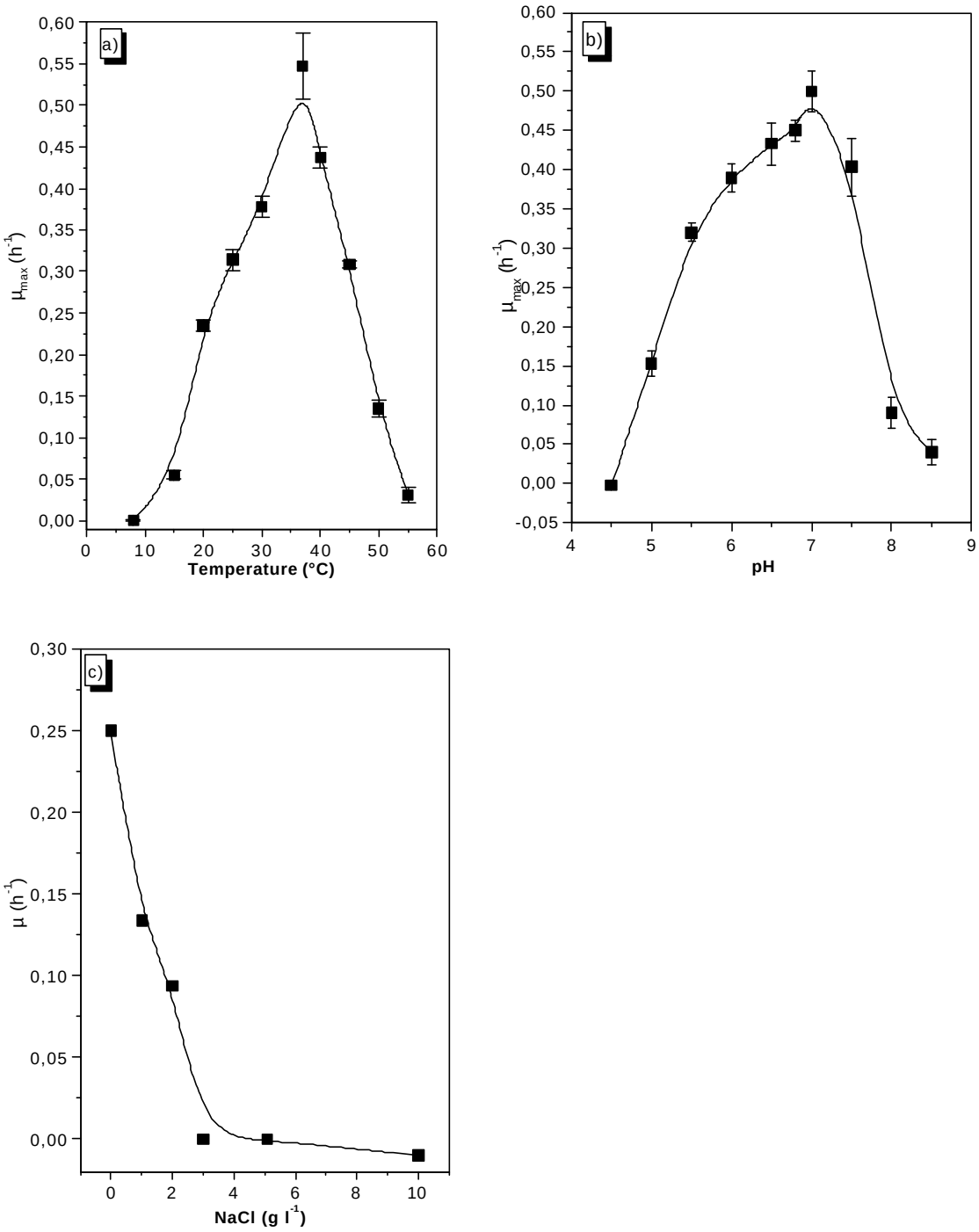

Fig. 9.3. Specific growth rates of strain KS SP 4/6 as function of: a) incubation temperature; b) pH-values at 37 °C; c) NaCl-content at 37 °C.

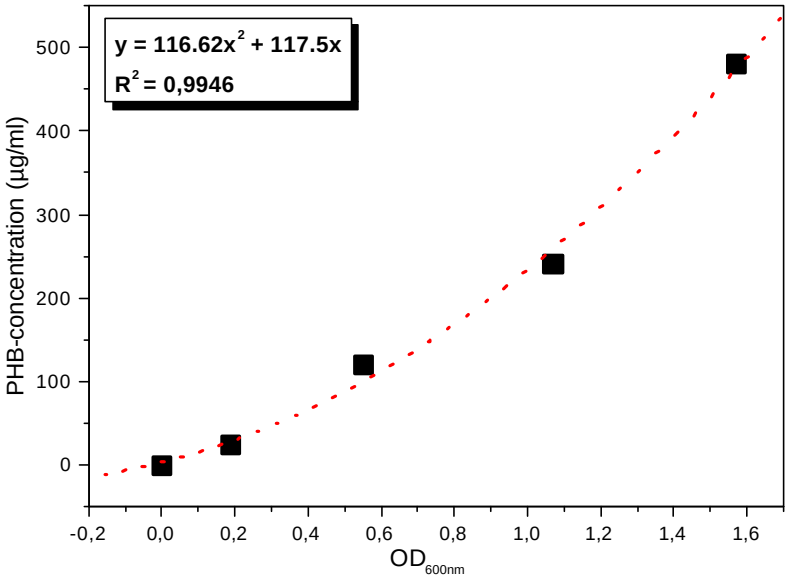

Fig. 9.4. Correlation between the measured OD_{600nm} and the measured PHB concentration of a stable PHB-suspension.